Studies on the pathogenicity of the entomopathogenic fungus Metarhizium spp. for the desert locust Schistocerca gregaria

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Studies on the Pathogenicity of the Entomopathogenic Fungus *Metarhizium* spp. for the Desert Locust *Schistocerca gregaria.*

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Dedicated to Roland, and our expected child.

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ABSTRACT

The entomopathogenic fungi *Metarhizium anisopliae* and *M. flavoviride* are being developed as mycoinsecticides for the control of the desert locust *Schistocerca gregaria*. An important part of this development is to identify possible virulence determinants. This study has investigated in detail steps in the penetration of host insect cuticle, and the production of toxins by the fungus.

Excised locust wings were used in a semi-*in vivo* technique to investigate germination, appressoria formation, penetration and protease production of 19 *M. anisopliae* and *M. flavoviride* isolates. Germination started at between 6 and 8 h after inoculation for all isolates, but 7 of them had not reached 50% germination by 12 h. Generally, the more pathogenic isolates initially produced only short germ tubes prior to appressoria formation, with the less pathogenic isolates producing longer, branched hyphae. Penetration of the wing occurred from the appressoria. A great range of protease production was seen for the isolates at the time of initial penetration. A significant correlation was found between the rate of germination and the median lethal time for the 19 isolates. No other significant correlations were found between the other parameters measured and virulence.

Destruxins are cyclic peptide lactone toxins isolated from *M. anisopliae*. A previous study has suggested a link between destruxin production and isolate virulence against the desert locust. The role of destruxins in fungal pathogenesis was investigated in particular with regard to their effect on the locust's excretory system. Destruxins A, A_2, B and E all inhibited fluid secretion in a dose dependent manner *in vitro* by Malpighian tubules of the desert locust *S. gregaria*. Experiments were conducted with ion channel blockers, inhibitors of cAMP phosphodiesterase and in calcium free conditions. From the results it is suggested that the inhibition by destruxin A of desert locust Malpighian tubule fluid secretion involves a cellular mechanism beyond the level of control by calcium or cAMP.

Destruxin A (25μg) inhibited the initial rate of removal of an injected dye, amaranth, from the haemolymph of freshly fed adult locusts indicating that destruxin A inhibits Malpighian tubule function *in vivo* in *S. gregaria* as well as *in vitro.*

Locusts on day 4 of infection with *M. anisopliae* isolate Me1 had a slower, reduced haemolymph circulation as indicated by the speed of spread of an injected dose of amaranth. Mycosed locusts were difficult to bleed and appeared to contain less haemolymph than controls. However, there was no significant difference in wet weight or the rate of water loss between control and infected locusts. This finding is discussed with regard to the involvement of destruxins in mycosis.

Destruxin A altered the ultrastructure of *S. gregaria* Malpighian tubules in the *in vitro* preparation used in the physiological experiments. Both control and destruxin treated tubules had changes in ultrastructure, that is vacuolisation and swelling of the organelles, consistent with slight anoxia due to the methods employed. Minor differences between control and destruxin-treated cells, however, were still apparent. It is concluded that destruxin inhibition of *S. gregaria* Malpighian tubule fluid secretion *in vivo* and *in vitro* is not the result of gross cellular damage. These observations suggest that destruxins act on the regulatory apparatus of the cell.

Haemolymph from locusts on day 4 of infection with *M. anisopliae* isolate Me1 was processed and analysed using an analytical RP-HPLC. No peaks were found to correspond with the 16 min retention time of the destruxin A standard. However, one or more substances present in fractions 15 and 16 (corresponding to retention times of 15 and 16 min) accelerated the heart rate of a semi-isolated *Manduca* larval heart preparation with an increase of 24% each. This translates to a level of 14-32 pg/μl of blood of presumptive destruxin A. This concentration of destruxin in the blood of mycosed locusts is a much lower level than that found by Samuels *et al.* (1988b) in *M. sexta* larvae. The reasons for this are discussed.

ABBREVIATIONS

cAMP	adenosine 3',5'-cyclic monophosphate
DMF	dimethyl formamide
DTNB	5-5'-dithiobis-(2nitrobenzoic acid)
ED_{50}	the 50% effective dose
EGTA	ethylene glycol-bis(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid
EPPS	N-(2-hydroxyethyl) piperazine-N'-(3-propane-sulfonic acid)
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	concentration required to give 50% inhibition
LD_{50}	dose required to give 50% kill
LT_{50}	time to 50% mortality
OD	optical density
PMSF	phenylmethylsulphonyl fluoride
ppm	parts per million
RH	relative humidity
RP-HPLC	reverse phase high performance liquid chromatography
SITS	4-acetamido-4'-isothio-cyanatostilbene-2,2'-disulphonic acid
spp.	species
ULV	ultra low volume
UV	ultra-violet
w/v	weight for volume

CONTENTS

CHAPTER 1. INTRODUCTION.

Entomopathogenic fungi.

At present chemical pesticides are the main agents used in crop protection, with, in 1988, less than 2% of the world pesticide market being biological products (Moore and Prior, 1993). There is increasing pressure, however, to move towards more environmentally friendly biological control systems due to the high costs of chemical pesticides, the occurrence of pest resistance, secondary pest outbreaks and the adverse effects of the chemicals on the environment and beneficial fauna (Hall and Papierok, 1982; Moore and Prior, 1993). Mycoinsecticides are safer than chemical insecticides in that they are selective, biodegradable, can be incorporated into IPM programs, and by remaining in the environment as successive generations they may give extended control (Moore and Prior, 1993).

Entomopathogenic fungi can gain entry into the insect in a number of ways: i) directly through the external cuticle, ii) through natural openings such as spiracles, iii) through wounds, or iv) via the digestive tract (Veen, 1966; McCauley *et al.*, 1968; Schabel, 1978). The majority of entomopathogenic fungi utilise the first route, direct entry through the external cuticle (McCauley *et al.*, 1968; Schabel, 1978). This makes them unique among insect pathogens, avoiding the necessity of having to be ingested and so they can be used against a wider range of insect pests (Hajek and St. Leger, 1994).

There are more than 700 species of fungi that are pathogenic to insects (Charnley, 1989). The Deuteromycotina contain the majority of entomopathogenic fungi, with *Metarhizium* and *Beauveria* being the best known genera as they have a wide geographical distribution and host range (Hall and Papierok, 1982; McCoy *et al.*, 1988). As early as the 1870's *Metarhizium anisopliae* was used to control the wheat cockchafer, *Anisoplia austriaca*, and the sugarbeet curculio, *Cleonus punctiventris*, in Russia, and has also been used successfully, as a conidial formulation 'Metaquino', to control spittlebug on sugarcane and in pastures in Brazil for nearly 8 years (McCoy *et al.*, 1988). *M. anisopliae* var. *major* has successfully

controlled the coconut pest, the rhinoceros beetle, *Orycetes rhinoceros* (McCoy *et al.*, 1988).

The life cycle of the fungus begins with the spore landing on the surface of the insect, and, provided the conditions are suitable, the spore will germinate. The germinated spore produces hyphae and then appressoria from which penetrant hyphae grow down through the cuticle, by a combination of mechanical pressure and enzymatic degradation (Charnley, 1984). The hyphae grow inwards until they penetrate the haemocoel, where hyphal bodies bud off and circulate around the rest of the body in the haemolymph (Prasertphon and Tanada, 1968). Further growth may occur within the host, although it is often confined to the haemolymph (Charnley, 1984). Eventually the insect dies and hyphae then emerge from the cuticle to sporulate on the surface, releasing spores to continue the cycle.

The infection process summarised above will only occur on specific hosts, that is, on insects whose potential sites of infection (e.g. the external cuticle) provide the correct conditions. This 'specificity' is influenced by a number of factors. These include microclimatic factors, spatiotemporal coincidence between the pathogen and the host and the physiological state of the host system (Boucias and Pendland, 1984).

It is unlikely that a single activity of the fungus alone is responsible for host death. There are a number of ways in which fungi are thought to either cause, or contribute to, the death of the host, although they have not all been experimentally verified. They include:

1) toxin production, either causing death directly or indirectly (Madelin, 1963; Roberts, 1981; Charnley, 1984),

2) profuse hyphal growth through tissues, chronically disrupting host physiology (Madelin, 1963; Charnley, 1984).

3) hyphal bodies causing the haemolymph to become viscous and stop circulating (Madelin, 1963; Charnley, 1984),

4) interfering with spiracles and tracheoles, either directly by profuse growth, or indirectly by destroying the nervous system, leading to asphyxiation (Madelin, 1963),

5) invasion of host tissue causing the release of autotoxicants (Madelin, 1963; Charnley, 1984),

6) starvation due to the cessation of feeding, or the fungus using up the host's soluble food reserves (Charnley, 1984),

7) indirectly due to other microbial infections entering the host through wounds caused by the fungus.

To establish an infection the fungus must successfully be able to attach to the cuticle, germinate, penetrate the cuticle and multiply within the insect. The attachment of the spore to the insect cuticle is seen to have two stages, the initial adsorption of the conidia to the cuticular surface via nonspecific passive interactions involving charged groups on both surfaces, followed by more specific attachment involving short-range stereochemical interactions (Fargues, 1984; Boucias and Pendland, 1991). Mucus and/or enzymes present on the conidia are thought to be involved in the second stage of attachment.

The conditions on the cuticle surface will then determine whether or not germination occurs. St. Leger *et al.* (1992) suggested that the nutritional requirements for germination and appressoria formation were frequently host related, with an insect's susceptibility or resistance to an individual isolate determined in part by the nutrient conditions on the cuticle prior to infection. Thinned skinned insects such as homoptera and lepidoptera had higher nutrient levels on their cuticles than hard cuticled insects such as coleopterans, therefore *M. anisopliae* isolates from coleopterans would germinate and produce appressoria in lower nutrient conditions than isolates from lepidopterans.

The presence of toxic or antifungal substances on the cuticle will also play a role in germination. Smith and Grula (1982) suggested a process of selective toxicity, involving short chain fatty acids in the presence of different nutrients. Inhibition of

germination of *Beauveria bassiana* in the presence of fatty acids, from the cuticle of *Heliothis zea*, was reversed by the addition of certain nutrients. Boucias and Latgé (1988), investigating the germination of *Conidiobolus obscurus* and *Nomuraea rileyi* on host and non-host cuticles, suggested that germ tube growth was stimulated by relatively nonspecific nutrients and that *in vivo* this was prevented on non-host cuticles by inhibitors or host components activated by the fungus. Latgé *et al.* (1987) compared aggressive and non-aggressive strains of *C. obscurus* and found that cuticular extracts of the aphid *Acyrthosiphon pisum* stimulated ballistospore germination of the aggressive but not the non-aggressive strains.

This difference in germination on host and non-host insects and between virulent and non-virulent strains has been studied with regard to virulence and pathogenicity. Hassan *et al.* (1989) showed that soaked conidia germinate more rapidly than unsoaked conidia, and that increased speed of germination increased the pathogenicity of those isolates. This led them to suggest that the speed and synchrony of germination and appressorial formation are limiting factors in pathogenesis. Though Drummond *et al.* (1987) were able to find no consistent correlation between pathogenicity and the rate of germination of *Verticillium lecanii* against the glasshouse whitefly *Trialeurodes vaporariorum*, Jackson *et al.* (1985) found that one of the traits significantly contributing to, or associated with, the expression of virulence of *V. lecanii* against the aphid *Macrosiphoniella sanborni* was a fast rate of germination. Al-Aidroos and Roberts (1978) also showed enhanced virulence of *M. anisopliae* isolates was possibly related to earlier germination on mosquito larvae.

Once germination has occurred the length of germ tube produced, prior to the production of an appressorium and penetration, varies greatly. Pekrul and Grula (1979) found a difference between pathogenic and non-pathogenic isolates of *B. bassiana* infecting the corn earworm *H. zea*. They found that highly pathogenic isolates produced short germ tubes and penetrated quickly, whereas low pathogenic isolates had errant growth of hyphae and much longer hyphae prior to penetration. Extensive hyphal growth prior to infection may not be a negative factor as Schabel

(1978) suggested that it may enhance invasiveness of *M. anisopliae* via synergism with other hyphae and the production of an appressorial complex. St. Leger *et al.* (1989) showed that, provided the conditions were suitable, *M. anisopliae* would produce appressoria *in vitro*, with hydrophobic surfaces being better at inducing differentiation than hydrophilic. They concluded that a thigmotropic response as well as a nutritional response were required for appressoria formation, as a minimum level of nutrients was essential with excess leading to undifferentiated mycellial growth.

Surface topography is also seen to influence the formation of appressoria. St. Leger *et al.* (1991b) found that *M. anisopliae* would form appressoria more readily on smoother surfaces like those of late instar tobacco hornworm, *Manduca sexta*, larvae, whereas on the cuticles of earlier instar larvae that were more convoluted there was more extensive hyphal growth prior to appressoria formation. The hardness of the surface may also affect penetration, as Pekrul and Grula (1979) found that although *B. bassiana* readily germinated on the hard surface of the head of the corn earworm no penetration was observed. Entomopathogenic fungi are often seen to penetrate the easier sites of softer cuticle at joints and between segments (Charnley, 1984).

The principal roles of the appressorium include attachment and production of infection pegs. The timing of appressoria formation and the time taken to penetrate the cuticle and invade the haemocoel could therefore be important factors of virulence. Intimately associated with these processes is the production of enzymes. Both the amounts produced and the infection stages at which they are produced could greatly affect the ability of the fungus to invade an insect. It seems reasonable that in order to aid infection the enzymes should be produced just prior to, and during the penetration process.

The involvement of enzymes in the penetration of insect cuticle has been studied by many authors. McCauley *et al.* (1968) found evidence of histolysis in the penetration of the cuticles of some species of elaterid larvae infected with *M. anisopliae*. Zacharuk (1970) put the disappearance of the lipid layer of the host integument under the infection cushions of *M. anisopliae* down to the action of

enzymes, and Hassan and Charnley (1989) found an absence of the lamellar structure in the cuticle of *M. sexta* in the vicinity of penetrating *M. anisopliae* hyphae and suggest this was due to enzyme degradation.

The insect cuticle is the main barrier against infection by fungal pathogens. Insect cuticles are generally composed of a thin outer epicuticle comprising lipids and proteins, and below that a thicker procuticle comprising chitin and proteins that in adult cuticle are often sclerotised (Andersen *et al.*, 1995). Cuticular chitin from different sources has minor differences in chain length or acetylation between insect groups, but proteins are known to be much more diverse. St. Leger *et al.* (1986a) determined the protein content of locust cuticle to be 73% of cuticular dry weight, with alanine and glycine being the principle amino acids of hard and soft cuticle respectively. They found that pretreating the cuticle with protease increased the ability of chitinase to degrade cuticle and so suggested that most of the chitin was masked by protein. They also found that sclerotised cuticle was relatively resistant to enzymolysis by fungal enzymes and suggested that mechanical pressure plays a greater role in penetration of sclerotised cuticle. Smith *et al.* (1981) also found that proteases followed by chitinases were necessary for cuticle degradation, in this case of *H. zea* 'ghosts'.

The cuticle-degrading enzymes of *M. anisopliae* have been extensively studied. When grown in liquid culture containing locust cuticle *M. anisopliae* is seen to produce extracellular cuticle-degrading enzymes rapidly and sequentially, with those of the proteolytic complex released first and the chitinases released much later (St. Leger *et al.*, 1986c). These proteolytic enzymes were seen to bind to cuticle nonspecifically to protein and chitin alike, but the chitinase seemed to bind irreversibly to chitin (St. Leger *et al.*, 1986b). This proteolytic complex has since been shown to comprise a chymoelastase PR1, a serine protease PR2, a cysteine protease PR4, a metalloproteinase, a carboxypeptidase and several amino peptidases (St. Leger *et al.*, 1987a, 1994a, 1994b; Cole *et al.*, 1993). PR1 has been shown to be the most effective enzyme against cuticle, with PR4 then PR2 having considerably

lower cuticle-degrading abilities (St. Leger *et al.*, 1987a; Cole *et al.*, 1993). PR1 and PR2 production on blowfly wings occurred at the same time as appressoria production, but no chitinase activity was seen, suggesting that chitinases may play a greater role in the saprophytic stage of the fungus (St. Leger *et al.*, 1987b). Goettel *et al.* (1989) used immunogold staining of ultra-thin sections of cuticle to show that PR1 was present at high levels on and around appressoria and penetration pegs of *M. anisopliae* infecting *M. sexta* larvae. The importance of PR1 in *M. anisopliae* mycosis was shown by St. Leger *et al.* (1988a) who found that an inhibitor of PR1, when applied during the infection of *M. sexta* larvae, significantly delayed mortality.

Extracellular enzymes capable of degrading lipids, proteins and/or chitin have been produced on solid media or liquid culture by a number of other entomopathogenic fungi, including *Cordyceps militaris*, *B. bassiana*, *Aspergillus flavus*, *N. rileyi* and *Entomophthora* species (Charnley, 1984). As with the cuticle-degrading enzymes of *M. anisopliae* their exact role in fungal pathogenesis has yet to be ascertained, but it would seem that proteolytic and chitinolytic activity are necessary for penetration of the cuticle. One of the proteases produced by *B. bassiana* has recently been shown to be very similar to *M. anisopliae* PR1, which led the author to suggest that such proteases are possibly widespread in entomopathogenic fungi (Joshi *et al.*, 1995). St. Leger *et al.* (1991a) suggest that many enzymes are important determinants of virulence by enabling the pathogen to exist with the changing metabolic processes associated with the host's diseased state.

Growth of penetrating *M. anisopliae* hyphae through the cuticle appears to vary. In some insects penetrant hyphae reach the body cavity by either direct or stepwise progression through the procuticle, and in other cases penetration plates or swellings are formed from which lateral branches develop in all directions (McCauley *et al.*, 1968; Schabel, 1978; St. Leger, 1991). In the desert locust, *Schistocerca gregaria*, Gunnarsson (1988) found that *M. anisopliae* had penetrated the epicuticle within 12 to 18 hours of inoculation, that penetrant hyphae grew beneath the epicuticle and then continued through the procuticle with substantial

horizontal growth in each of the exo- and endocuticular layers. Penetrant hyphae are seen to grow along the cuticular lamellae, the path of least resistance (Prasertphon and Tanada, 1968; Schabel, 1978; Hassan and Charnley, 1989). Once within the body cavity hyphal bodies bud off from the hyphal tips and circulate within the haemolymph and within 36 hours of inoculation they were found in regions remote from the site of initial inoculation on *Galleria mellonella* larvae (Prasertphon and Tanada, 1968)

The first reaction of the insect to the invading fungus, in many cases, is a melanization of the cuticle (Prasertphon and Tanada, 1968; Schabel, 1978; Gunnarsson, 1988). This reaction is thought to be effective against weak or nonpathogenic organisms, but it appears to occur too late or in insufficient magnitude to stop fast growing or highly pathogenic isolates (Hajek and St. Leger, 1994). PR1 is seen to be able to hydrolyse melanized cuticle (St. Leger *et al.*, 1988b).

The main reaction in the cellular antifungal defence mechanism is encapsulation. Haemocytes are attracted to the site of fungal invasion, often before the fungus has reached the inner layers of the cuticle, where they attach to and aggregate on the basement membrane of the epidermal cells, becoming more spread and forming pseudopodia (Gunnarsson, 1988). The resulting granuloma is, again, only effective against weak pathogens, with virulent pathogens overcoming encapsulation and continuing to grow (Hajek and St. Leger, 1994). Phagocytes are also recruited to the site of infection, but phagocytosis does not always occur (McCauley *et al.*, 1968). Humoral immunity also plays a role in protecting the insect against fungal attack, but the exact mechanisms employed are not fully understood. Fungitoxic protease inhibitors and other antifungal factors are thought to be present in the haemolymph and may prevent lethal infections (Hajek and St. Leger, 1994). The plant food eaten by an insect host may also affect the ability of the fungus to grow within that host as certain foods may contain fungistatic secondary plant chemicals that remain active within the insect (Hajek and St. Leger, 1994).

Fungal Toxins : Destruxins.

A range of low molecular weight toxins has been identified from culture filtrates of a number of entomopathogenic fungi, but most have not been isolated from mycosed insects, and so their role in fungal pathogenesis is not fully understood (Charnley, 1984). Among the symptoms seen in insects that may implicate toxins in mycosis are limited growth of the fungal pathogen within the body of the host at the time of death (Charnley, 1984), sluggishness and lack of co-ordination just prior to death and the ability of the fungus to rapidly overcome parts of the immune system of the insect such as cyst formation and phagocytosis (Ferron, 1978). Yoder (1980) classifies pathogen-produced toxins into two types, those that are required for pathogenicity, i.e. are required for disease to occur, and those that are required for virulence, i.e. are required for a portion of disease development.

Some of the fungi known to produce toxins are *Beauveria*, *Cordyceps*, *Entomophthora*, *Metarhizium* and *Paecilomyces* (Ferron, 1978). *Entomophthora virulenta* was found to produce azoxybenzenoid compounds that were toxic to various Diptera upon injection (Gillespie and Claydon, 1989), and cordycepin was the toxin isolated from *Cordyceps militaris* (Ferron, 1978). A cyclic depsipeptide, beauvericin, is produced by *B. bassiana* and *Paecilomyces fumosoroseus*, and was found to affect the form, reactions and migrations of cultured insect cells (Ferron, 1978). *B. bassiana* also produces two other toxins, beauverolides and bassianolide. *M. anisopliae* was found to produce destruxins (Kodaira, 1961). *M. flavoviride* has also been shown to produce toxic compounds, the viridoxins (Gupta *et al.*, 1993).

Destruxins are a family of cyclic peptide lactone toxins, whose basic structure consists of 5 amino acids and a hydroxy acid (Tamura and Takahashi, 1971). Kodaira (1961; 1962) first isolated destruxins A and B from *M. anisopliae* culture filtrates and found that they were toxic to silkworm larvae, *Bombyx mori*, by injection. Since that original discovery a further 18 related molecules have been found (Suzuki *et al.*, 1970; Pais *et al.*, 1981; Gupta *et al.*, 1989; Wahlman and Davidson, 1993).

Many entomopathogenic fungi produce toxins (Charnley, 1984) but interestingly destruxins are the only fungal toxins that have been detected in moribund insects in sufficient quantities to cause death (Suzuki *et al.*, 1971). In lepidopteran larvae intrahaemocoelic injection of destruxins causes immediate tetanic paralysis, due to sustained muscle contraction, followed by a period of flaccid paralysis, in *G. mellonella, B. mori* and *M. sexta* (Roberts, 1966 and 1981; Samuels *et al.*, 1988b). The duration of this paralysis is dose dependent, with high doses being lethal. However the effects of destruxins on other insect orders are very different. Samuels *et al.* (1988a) found the dipteran *Calliphora vomitoria* to be susceptible to injected destruxins as adults but not as larvae. *S. gregaria* adults were unaffected by doses of injected destruxins sufficient to kill *M. sexta* larvae (Samuels *et al.*, 1988a), and Roberts (1981) states that although Orthoptera and Coleoptera are susceptible to injections of destruxins A and B they do not respond with overall tetanus.

Variations in susceptibility are also seen at the cellular level. Working with insect cell lines, Quiot *et al.*(1985) found that destruxins had an effect on both the *Gromphadorhina laevigata* and *B. mori* cell lines. These effects were still evident in the *Bombyx* cell line at a concentration that no longer had any effect on the *Gromphadorhina* cell line. Differences in susceptibility in both whole insects and isolated cell lines could be due to differing modes of action of destruxins in the various insect species, or a differing ability of the insects to detoxify the toxins.

The effects of destruxins on different tissue and cell types in a range of insect species have been investigated in order to establish both the role of destruxins in fungal pathogenesis, and their mode of action.

At the ultrastructural level destruxins caused the cells of the *G. laevigata* cell line to become highly vacuolized due to dilation of the endoplasmic reticulum (Quiot *et al.*, 1985). The mitochondria were dilated, their cristae changed, and in the nucleus pycnosis had occurred. DNA, RNA, and protein synthesis were also seen to be inhibited by destruxins (Quiot *et al.*, 1985). At the whole cell level Kershaw (1993) found that destruxins caused a reduction in cell viability and an inhibition of cell

replication in a *Spodoptera frugiperda* cell line. Additionally, destruxins have been shown to inhibit cell multiplication in other cell lines (Quiot *et al.*, 1985) in leukemic cell cultures (Odier *et al.*, 1992) and also to inhibit baculovirus replication (Quiot *et al.*, 1985).

Destruxins were first noted to have immunodepressive effects by Vey *et al.* (1985). They were shown to cause states of increased susceptibility to infection in *G. mellonella* larvae due to inhibition of multicellular encapsulation. Huxham *et al.* (1989) described the immunosuppressive activity of destruxins in Orthoptera in terms of a suppression of the stimulatory effects of ß1,3-glucans. Cerenius *et al.* (1990) suggested instead, that the observed inhibition of ß1,3-glucan mediated activation of phenoloxidase by destruxins was actually a result of destruxin-induced degranulation of the haemocytes. Degranulation of insect haemocytes would deplete them of stored components of the prophenoloxidase activating system, reducing the effectiveness of the insect's immunological defences. This toxin-induced immunosuppressive activity could be beneficial to an entomopathogen *in vivo*.

Destruxins were seen to have physiological and structural effects on the Malpighian tubules of adult *C. vomitoria* (S.R. Watkins, J.S. Cook and A.K. Charnley, unpublished). The rate of removal of amaranth from the haemolymph of adult blowflies was inhibited by destruxins, and this inhibition was dose dependent. Destruxins were also shown to cause disruption of the cell structure in the Malpighian tubule cells, with extensive vacuolisation of the cytoplasm, the breakdown of the basal infolds and swollen mitochondria. Similar results were observed by Dumas *et al.* (1994) in *G. mellonella* larvae 24 hours after intrahaemocoelic injection of destruxins A and E.

To investigate the role of destruxins in fungal pathogenesis Samuels *et al.* (1988a) compared three *M. anisopliae* isolates. One, Me1, produced large quantities of destruxins *in vitro*, the other two produced much less. Me1 was the most virulent isolate, with the lowest LT_{50} for *M. sexta*, and caused paralysis of the host prior to death, something not seen with the other two isolates. Me1-infected *M. sexta* had far

fewer hyphal bodies in the haemolymph 24 hours prior to death than the haemolymph of larvae infected with either of the other two isolates, which, in each of the latter cases, was packed with hyphal bodies at that time. Haemolymph from the infected insects was extracted and analysed for the presence of destruxins, and only in Me1 infected insects were any destruxins seen. It was suggested that destruxins could have one of two roles, a pathogenic role where the toxins are active in causing disease, or an aggressive role where they facilitate the establishment of the pathogen.

Kershaw (1993) compared the *in vitro* destruxin production of a range of *M. anisopliae* and *M. flavoviride* isolates with the virulence of these isolates to particular host species. Although no distinct correlation was seen, a large number of those isolates pathogenic to *M. sexta* also produced destruxins, which, as Kershaw (1993) states, is consistent with the view that toxicosis plays a part in the pathogenesis of lepidopteran insects. There were isolates however, that had a low LT_{50} in *M. sexta* but did not produce destruxins *in vitro*, demonstrating that entomopathogenic fungi can be highly pathogenic without the aid of destruxins (Kershaw, 1993). Many *M. flavoviride* isolates are pathogenic to orthopteran hosts but do not appear to produce destruxins (Kershaw, 1993), although this species of fungus produces an alternative group of toxins, the viridoxins (Gupta *et al.*, 1993).

The mode of action of destruxins has also been investigated. Samuels *et al.* (1988b) showed that the sustained muscle contractions causing paralysis in *M. sexta* larvae after injection of destruxins could be abolished using calcium channel blockers ($CdCl_2$ and nifedipine). They went on to suggest that destruxins were causing the depolarisation of muscle membrane seen by either directly or indirectly opening calcium channels. This is consistent with the subsequent finding of Cerenius *et al.* (1990), who showed that destruxin E induced degranulation of crayfish haemocytes required the presence of calcium. The same workers found that the anion channel blocker SITS also prevented destruxin induced degranulation. It is possible to explain this observation in terms of a requirement for the presence of open anion channels to

allow the influx of chloride counterions when destruxin causes the opening of calcium channels.

Bradfisch and Harmer (1990) also showed destruxin B caused a rapid depolarisation of the muscle membrane in *Heliothis virescens*, again suggesting the activation of a voltage dependent calcium channel. This is in keeping with the findings of Samuels *et al.* (1988b), although Bradfisch and Harmer (1990) did suggest that the destruxin activated channel was not normally associated with the active membrane responses to synaptic activation.

While the work described above suggests that destruxin action involves Ca^{2+} channels, the results of Sloman and Reynolds (1993) do not fit in with this mode of action for destruxins. Destruxins were shown to inhibit basal and stimulated levels of secretion of ecdysteroid from *M. sexta* prothoracic glands *in vitro*. Cyclic AMP- and calcium-elevating treatments stimulate ecdysteroid secretion, therefore it would be expected that destruxin treatment would lead to calcium entry and a resulting stimulation of ecdysteroid secretion. This was not the case. Destruxins were seen to inhibit ecdysteroid secretion, and it was suggested that in this case destruxins may act at a level beyond that of the second messengers cAMP and calcium.

The relative toxicities of individual pure destruxins has been tested by a number of authors, but the use of different criteria to determine toxicity, different application methods and different insect species tested has lead to some confusion. Kodaira (1961) was the first to test the toxicity of injected destruxins A and B to *B. mori* larvae. He found between 0.014 and 0.03 μg/g was needed for either destruxin A or B to have an effect on larval behaviour, and a concentration of about 0.3 μg/g was needed to cause paralysis and death. This agreed with Tamura and Takahashi (1971) who found that the minimum amount of destruxin needed to cause paralysis and death after injection into *B. mori* larvae was 0.28 μg/g for destruxin A and 0.34 μg/g for destruxin B. Suzuki (1979) found much higher concentrations of both destruxin A and B (1.5 μg/g in both cases) were needed to cause paralysis followed by recovery after injection into *B. mori* larvae.

G. mellonella larvae were used to conduct dose-mortality experiments on injected destruxins (Fargues *et al.*, 1986). They showed that destruxins E and A had very similar LD_{50} values (49.3 and 49.9 μg/g respectively), with destruxin B having a much higher LD_{50} (130 μg/g). The *per os* effects were similar. The cytotoxic effects of these three destruxins were compared in *G. mellonella* larvae (Vey and Quiot, 1989), and destruxin E was seen to be more toxic than destruxin A which was in turn more toxic than destruxin B. Dumas *et al.* (1994) also found the injected and *per os* effects of destruxins on *G. mellonella* to be very similar. They also showed destruxin E to have the greatest cytotoxic activity of the destruxins and destruxin analogues tested, but destruxin A and destruxin E were not significantly different in their lethal activity toward *G. mellonella* larvae.

Kershaw (1993) investigated the effects of different destruxins on another lepidopteran, *M. sexta*. Destruxins A and E had similar ED_{50}'s (0.25 μg/g), measured as an inability of the larvae to right themselves 1 minute after injection of the destruxins. Destruxins A_2 and B were less effective having ED_{50}'s of 0.3 and 0.37 μg/g respectively. The destruxins were seen to be extremely effective cardioaccelerators when tested on a semi-isolated *M. sexta* larval heart preparation, with destruxins A, B, A_2 and E all being effective at 10 ng/μl, but there were no apparent differences between these destruxins.

Kershaw (1993) also investigated the effects of individual destruxins on a *S. frugiperda* ovarian cell line (Sf9). Destruxin E caused a 50% reduction in cell numbers at concentrations of between 0.0625 to 0.125 μg/ml, but destruxin A required 5 times that concentration to produce the same result, and destruxins A_2 and B even higher concentrations. This is the same order of toxicity found by Quiot *et al.* (1985) working with a *B. mori* cell line. They showed that destruxin E produced effects that were evident at 0.05 ppm, but destruxin A needed 0.25 ppm to produce even weak effects, with destruxin B having no effect at 0.5 ppm. Very similar results were obtained by Dumas *et al.* (1994) who found the threshold of activity in *in vitro* *B. mori* cell cultures to be 0.20 μg/ml for destruxin A and 0.05 μg/ml for destruxin E.

Not only have different levels of activity been seen with different destruxins, but also different cytotoxic effects. Quiot *et al.* (1985) showed that with destruxins A and B the cells of the *B. mori* and *G. laevigata* cell lines were contracted and granular in appearance, whereas with destruxin E they agglomerated and floated in the medium. Destruxins A and E were shown to have different cytotoxic effects on the Malpighian tubules of *G. mellonella* larvae (Dumas *et al.*, 1994). Destruxin A caused swollen mitochondria and the formation of vesicles within the microvilli, but destruxin E lead to strong alterations at the level of the nucleus and endoplasmic reticulum with migration of cellular components to the lumen of the tubule. This may suggest a different mode of action for some destruxins, although Quiot *et al.* (1985) suggest it is more likely to be a difference of 'intensity of action'. Dumas *et al.* (1994) noted that the most active destruxins were often the most hydrophobic, and suggested this was possibly due to their higher ability to penetrate cells through lipoproteic components of the cell membranes.

The fate of injected destruxins has not been greatly studied but is an area of much interest, particularly with respect to the various susceptibilities of the different insect orders to destruxins. Increased susceptibility may be linked with a reduced ability to detoxify destruxins. The reversible nature of the destruxin induced paralysis of *M. sexta* larvae (Samuels *et al.*, 1988b) indicates a possible detoxification process. Differences in the rate of removal of an injected dose of destruxins from the haemolymph of two Lepidopteran larvae, *M. sexta* and *B. mori* (Samuels *et al.*, 1988a) may be accounted for by a greater ability of *M. sexta* to detoxify destruxins.

In vitro incubation of destruxins in *M. sexta* and *B. mori* haemolymph has been carried out and over a 24 hour period and no decline in the destruxin titre occurred (Samuels, 1986). The same result was seen in *G. mellonella* (Jegorov *et al.*, 1992) and *Locusta migratoria* (Cherton *et al.*, 1991) haemolymph over a 3 hour period.

In *G. mellonella*, injected [^{3}H]-dihydrodestruxin reaches a maximum in the haemolymph after 1hr (Jegorov *et al.*, 1992). By 2 hours there is greater activity in

the hindgut and midgut, and as Jegorov *et al.* (1992) suggest this may explain why midgut and circulating haemocytes are the main organs and tissues attacked by destruxins (Vey and Quiot, 1989). This is not seen in locusts. Cherton *et al.* (1991) and Loutelier *et al.* (1994) both showed a peak in the level of injected destruxins in the haemolymph well before 1 hour, and in fact a noticeable decrease in the destruxin level had already occurred by 1 hour. A hydrolysis product of destruxin E, E-dioldestruxin, was noted to be at a maximum level in the haemolymph by 1 hour after the injection of destruxin E (Loutelier *et al.*, 1994). This difference between the two insects may indicate a more rapid or greater detoxifying ability in locusts than in *G. mellonella.*

Jegorov *et al.* (1992) showed that *G. mellonella* larvae did have the ability to detoxify destruxin to a linear product, with almost all the injected destruxin broken down, in all of the body parts observed, within 3 hours. The highest level of this product could be seen in the haemolymph 2 hours after injection of destruxin.

Cherton *et al.* (1991) suggest that the haemolymph probably acts as a simple carrier of the toxin and its detoxified products to and from the sites of detoxification. This was because of the lack of any *in vitro* breakdown of destruxins, mentioned previously. However, in some insects the haemolymph is an important site of neuropeptide degradation. In *M. sexta Manduca* adipokinetic hormone was inactivated by an enzyme present in the plasma (Fox and Reynolds, 1991), and in cockroaches the haemolymph was shown to contain enzymes capable of hydrolysing proctolin (Steele and Starratt, 1985). In locusts, however, it would appear that the Malpighian tubules and the ganglia play a greater role in neuropeptide degradation than the haemolymph (Siegert and Mordue, 1987; Isaac, 1988). These studies show the presence of peptidases in the blood and tissues of some insects that could be involved in the rapid detoxification of destruxins.

Cherton *et al.* (1991) and Loutelier *et al.* (1994) followed the distribution of injected destruxins A and E and a number of resulting detoxified products in certain tissues of adult *L. migratoria.* They used a direct method, thus avoiding any losses

due to extraction processes. They found that both destruxins A and E were rapidly transported round the body, with maximum levels reached, in the tissues tested, at or before 30 minutes post-injection. By 1 hour some detoxification had occurred, with generally lower levels of destruxins A and E, and higher levels of E-dioldestruxin, than at 10 or 30 minutes. Cherton *et al.* (1991) suggested from these findings that the fat body played a role in both storage and detoxification, with the pericardial tissue also carrying out detoxification. The presence of both destruxin E and E-dioldestruxin in the Malpighian tubules, and a decrease in the level of both of these over time, pointed to the involvement of these tissues in an excretory process. The results of Loutelier *et al.* (1994) would seem to confirm this. They also went on to show that another detoxification process was occurring, the conjugation of destruxin E with glutathione, this then leading to a cysteine-destruxin E conjugate. No linear detoxification products were observed.

Loutelier *et al.* (1995) observed the *in vivo* detoxification of destruxin A in *L. migratoria* and found the detoxification process to be different for that of destruxin E. A linear detoxification product of destruxin A was found, resulting from the ring-opening of the lactone function. A linear molecule resulting from the opening of the ring structure of destruxin A has been shown to have no toxic activity (Dumas *et al.*, 1994).

Malpighian tubules.

The Malpighian tubules of insects are long, thin blindly ending tubes that arise from the gut at the junction between the midgut and the hindgut, via a series of ampullae (Bell and Anstee, 1977). Adult *S. gregaria* posses about 230 Malpighian tubules with roughly one third directed anteriorly, attaching distally at the caeca, and the remainder directed posteriorly, attaching to other tubules, the rectum or large tracheae (Garrett *et al.*, 1988). The tubules lie free in the haemocoel and each has muscles wound helically around it , as well as a rich network of tracheoles associated with it (Bell and Anstee, 1977; Garrett *et al.*, 1988).

Three morphologically distinct regions of the tubules are seen, the distal, middle and proximal regions, with the middle region being the longest (Garrett *et al.*, 1988). The tubules are all one cell thick and in *L. migratoria* and *S. gregaria* a number of different cell types have been seen over the different regions. These include principle cells, stellate cells, mucocytes and granulocytes, although the exact role of each of these cell types is still not clear (Bell and Anstee, 1977; Charnley, 1982; Garrett *et al.*, 1988; Prado *et al.*, 1992).

The Malpighian tubules are part of the insect's excretory system, whose role in water balance in the locust is a balance between urine production by the Malpighian tubules and reabsorption by the rectal wall (Mordue, 1969). The rate of urine production by the Malpighian tubules is known to be under the control of diuretic factors, but in locusts there is some controversy over the number of diuretic factors involved (Spring, 1990). There is known to be a diuretic peptide in *L. migratoria* which acts via the second messenger cAMP (Morgan and Mordue, 1985), but it appears that the effects of at least some diuretic factors are mediated by more than one second messenger (Spring, 1990).

The Malpighian tubules produce an isosmotic filtrate of the haemolymph, driven by the active transport of ions into the tubule lumen (Bradley, 1985). The fluid in the tubules, known as primary urine, carries excretory wastes, toxic compounds and excess ions for excretion, and also sugars and amino acids which, along with various ions, can be selectively reabsorbed via active transport mechanisms in the hindgut (Bradley, 1985). Water flow is driven by solute-water coupling at the highly folded apical and basal membranes, while the movement of non-transported organic molecules is extracellular, via intercellular junctional spaces (Bradley, 1985).

Potassium, and associated counter ions, create an osmotic gradient, drawing water through the epithelium. At the basal membrane, potassium, sodium and chloride ions are known to enter the cell (Nicolson, 1993). Potassium is thought to enter passively through potassium channels, and sodium and chloride ions were thought to enter through electroneutral cotransport systems (Baldrick *et al.*, 1988),

but Fogg *et al.* (1993) argue against this in *L. migratoria*. At the apical membrane a proton pump and associated K/H and/or Na/H antiporter actively transport potassium and some sodium ions into the tubule lumen, with chloride ions passively crossing the membrane through chloride channels (Nicolson, 1993). Active transport mechanisms are found in the tubules for removing highly toxic compounds, or common metabolic waste products (Bradley, 1985). It is thought that the speed with which these compounds, and tested dyes, are cleared from the insect is not related to molecular weight but to the presence of strongly acidic groups on the molecule.

Locusts.

Schistocerca gregaria (Forsk.), the desert locust is thought to be the most dangerous migratory locust species (Brader, 1988). Locusts occur in two phases, a 'solitarious' phase where they shun company and move relatively little, mostly as adults flying at night, and a 'gregarious' phase where they are attracted to one another, forming large, high density populations which move virtually continuously by day, both as marching juveniles and as flying adults (Haskell, 1992). Locusts are only a problem in the gregarious phase where they can form swarms of 20-50 million locusts per sq km, and cover up to 100 sq km, with each locust able to consume its own body weight in food a day (Courshee, 1990). Man has been plagued by such locust swarms for about 9000 years, and good rain after a drought is thought to be a key factor in setting off a plague (Brader, 1988). Costs of the latest *S. gregaria* plague control were US$ 150 million (Brader, 1988).

Mycoinsecticides.

In the past mycoinsecticides have been unsuccessful due to unreliable field performance, false expectations and interpretation of field results, high production costs and poor storage properties (Moore and Prior, 1993). The attributes required of a fungal pathogen are 1) good production features, which include high sporulation, optimum utilisation of available substrate and genetic stability, and 2) good field

performance, which includes a high virulence to the target and a suitable host range (Prior, 1992). In the search for a fungal pathogen the question arises as to whether to choose a pathogen that has an old association with the host and therefore will have a high specificity but lower virulence, or a new association that may have a high virulence and so have a dramatic effect on the host but may not be specific enough (Prior, 1992).

The advances in molecular biology to date allow for the possibility of strain improvement through genetic modifications, either by parasexual recombination or direct gene manipulation (Clarkson, 1992; Moore and Prior, 1993). Once the gene or genes for virulence have been identified the number of copies could be increased to increase the virulence of the isolate, or these genes could be moved form one isolate to another to give greater virulence to an isolate with other beneficial attributes but a lower virulence (Clarkson, 1992). Genetic modifications may also allow the improvement of specificity, resistance to adverse environmental conditions and agricultural factors such as fungicides (Moore and Prior, 1993). Another important aspect of the use of molecular biology in strain improvement is the ability to 'fingerprint' isolates below the variety level and so distinguish between isolates introduced into the field in control programs and those naturally occurring, and also avoid any overlap in the development of fungal pathogens (Clarkson, 1992; Moore and Prior, 1993).

The formulation of mycoinsecticides must also be reformed in order to be able to utilise any advances in strain improvement. Bateman *et al.* (1993) showed that *M. flavoviride* (whose spores are lipophilic and therefore suspend readily in oils) had enhanced infectivity towards locusts in an oil formulation as compared with a water based formulation, and that infection post-application was not dependent on high humidity. This increased infectivity in oil (observed in this case using *B. bassiana* against the cocoa weevil) is suggested by Prior *et al.* (1988) to be due to greater adhesivness to the lipophilic insect cuticle. The drying of the conidia prior to field application (McClatchie *et al.*, 1994) and the addition of a sunscreen to the

formulation to protect against the harmful effects of UV light in the field (Moore *et al.*, 1993) can both increase the effectiveness of mycopathogens in high temperature conditions such as those in which the desert locust are found.

One limiting factor of fungal pathogens is their slow speed of kill in comparison with chemical pesticides. However, Moore *et al.* (1992) showed that crop protection may be effected prior to host death because *M. flavoviride* infected locusts showed a reduction in feeding over a five day period. As the locust is a pest because it eats this is an important consideration.

Oil formulations of *Metarhizium* and other Deuteromycotina can be sprayed onto the target pest using existing ultra low volume controlled droplet application machinery, making them a good choice of mycopathogen (Bateman, 1991). The proven safety record and relative ease of production on simple culture medium of these fungi also make them good candidates for mycoinsecticides (Prior, 1992). Another possible way of increasing their effectiveness may be to combine several different virulent isolates in a single formulation, as this may have a greater effect on the host by challenging it with a wider range of virulence determinants (Prior, 1992). This stratagem was adopted in Microgermin® produced by Chr. Hansen's Biocontrol Systems for control of glasshouse aphids.

Pathogenicity of a fungal pathogen is dependant on a complex relationship between the ability of the fungus to invade and the ability of the host to prevent it (Huxham *et al.*, 1989). Pathogenicity is a qualitative term, defined as the ability to cause disease, whereas virulence is a quantitative measure of the infectivity and severity of the disease (Read, 1994).

M. anisopliae is known to infect insects of the orders Lepidoptera, Coleoptera and Orthoptera (Roberts, 1981), and a wide range of virulence levels are found in *Metarhizium* species, but the reasons for these differences are not known. *Metarhizium* species are being developed as control agents of locusts and grasshoppers under a collaborative research programme between the International Institute of Biological Control (IIBC), the International Institute of Tropical

Agriculture (IITA) at its Biological Control Centre for Africa station in Cotonou, Republic of Benin, and the Departement de Formation en Protection des Vegetaux (DFPV) in Niger (Prior *et al.*, 1992). Therefore it is important to try to identify the factors that confer virulence among isolates. This information will be useful in rational strain selection and strain improvement programmes, using parasexual recombination of isolates with different attributes and/or direct genetic manipulation.

Aims of the Project.

In this study a semi-*in vivo* technique was employed in an attempt to identify virulence factors involved in the penetration of *S. gregaria* cuticle by *Metarhizium* spp. (see chapter 1). Previous attempts, with other insects, to link, in particular, cuticle-degrading enzymes with virulence have involved monitoring enzyme production *in vitro* in liquid culture or on agar plates. Such studies have only limited relevance to the process of cuticle penetration.

Destruxins have been linked with virulence towards the vine weevil, *Otiorrynchus sulcatus*, the tobacco hornworm, *M. sexta*, and to a lesser extent the desert locust, *S. gregaria* (Kershaw, 1993). Therefore an additional object of the present work was to determine the possible role of destruxins in the pathogenesis of *M. anisopliae* for the desert locust. The excretory organs, Malpighian tubules, were the focus for this work because previous studies have established their susceptibility to destruxins (see chapters 3 to 6).

CHAPTER 2. FACTORS INVOLVED IN THE PENETRATION OF *SCHISTOCERCA GREGARIA* CUTICLE BY *METARHIZIUM* SPP.

INTRODUCTION

One important aspect of a good mycoinsecticide is a high virulence to the target pest. *Metarhizium* spp. are being developed as part of a collaborative project (IIBC/IITA/DFPV) for the control of the desert locust, *S. gregaria* (Prior *et al.*, 1992). A wide range of virulence levels are found in these *Metarhizium* spp. but the reason for this is not known. In order to utilise these entomopathogenic fungi to their full potential it is necessary to understand the factors involved in conferring virulence to the fungal pathogens.

In this chapter a quantitative approach is taken to try to identify potential virulence determinants. Aspects of the penetration process, i.e. germination, appressorium formation, penetration and protease production, on a semi-*in vivo* locust wing preparation, are measured in an attempt to correlate these parameters with virulence to the desert locust.

MATERIALS AND METHODS

Spore germination, appressorium formation and penetration on excised locust wings.

Spore germination, appressorium formation and penetration of the 19 *Metarhizium anisopliae* and *flavoviride* isolates listed in Table 1 were studied using excised locust wings. The wings were removed from adult locusts 3 to 7 days after ecdysis (see Appendix I for locust rearing conditions) and surface sterilised. This involved the wings being placed in a Petri dish lid inside a polythene bag. Approximately 25 ml propylene oxide was poured into the Petri dish base and this placed adjacent to the lid in the bag. The bag was then sealed and left overnight, leaving the propylene oxide to evaporate and so expose the wings to an atmosphere of propylene oxide. The sterilised wings were then spread flat on 2% water agar plates, as shown in Fig. 1. The wings were inoculated by spraying a spore suspension of 10^7 spores/ml directly onto the wing using a Quick-fit glass fine spray apparatus with a rubber hand pump. Lids were placed on the water agar Petri dishes and the preparations incubated at 27°C until observations were made.

Spore suspensions and viability.

Spores were harvested in 0.04% Tween 80 from $^1/_4$ strength SDA (Sabouraud's Dextrose Agar) plates grown at 27°C for 10 to 14 days. The spore solution was sonicated for 20 min and then filtered through 4 layers of muslin. Spore concentration was determined using a haemocytometer, and appropriate dilutions made to give a concentration of 10^7 spores/ml. Isolates used were grown from stock plates spread every three months from stock solutions of spores in 10% glycerol stored at -20°C. The stock plates were kept at 4°C.

All spore suspensions used were tested for spore viability. The agar slide technique used was adapted from that developed by Hall (1977). SDA was spread into three small pools on a glass microscope slide using a Pasteur pipette. 0.01 ml of the spore suspension, diluted with 0.04% Tween 80 to 0.5 x 10^6 spores/ml, was spread

Table 1. Origin of *Metarhizium anisopliae* and *M. flavoviride* isolates used in experiments.

ISOLATE	*METARHIZIUM SPP.*	COUNTRY	HOST
Arsef 324	*M. anisopliae*	Australia	Orthoptera: Acrididae
299984	*M. anisopliae*	Trinidad	Hemiptera: Cercopidae
324673	*M. flavoviride*	Tanzania	Orthoptera: Oypgomorphidae
298059	*M. anisopliae*	New Guinea	Coleoptera: Scarabaeoidae
299981	*M. anisopliae*	Trinidad	Hemiptera: Cercopidae
168777ii	*M. anisopliae*	Ethiopia	Orthoptera: Acrididae
152222	*M. anisopliae*	India	Coleoptera: Curculionidae
Arsef 2023	*M. flavoviride*	Galapagos Is.	Orthoptera: Acrididae
Arsef 727	*M. anisopliae*	Brazil	Orthoptera: Tettigonidae
I91 676	*M. anisopliae*	Pakistan	Orthoptera: Acrididae
Arsef 438	*M. anisopliae*	Australia	Orthoptera: Gryllidae
Arsef 440	*M. anisopliae*	Australia	Orthoptera: Gryllidae
Arsef 439	*M. anisopliae*	Australia	Orthoptera: Gryllidae
298061	*M. anisopliae*	New Guinea	Coleoptera: Hispidae
Me1	*M. anisopliae*	Brazil	Coleoptera
Nr 48	*M. anisopliae*	Thailand	Orthoptera
330189	*M. flavoviride*	Niger	Orthoptera: Acrididae
I90 574	*M. anisopliae*	Pakistan	Orthoptera: Acrididae
I91 633	*M. anisopliae*	Oman	Orthoptera: Gryllidae

Figure 1. Three surface sterilised locust wings spread flat on a 2% water agar plate.

onto each pool of agar then the slide allowed to dry for 5 min. The slide was placed on three Whatman No. 1 filter papers moistened with distilled water in the lid of a 9 cm Petri dish. The base was then placed on the lid and it was incubated at 27°C. Germination was assessed between 12 and 24 h after inoculation. 100 spores were counted per pool and viability expressed as the mean germination percentage of the 300 spores. Only those spore suspensions that reached greater than 95% germination were used. Germination was deemed to have occurred when a germ tube was greater than or equal to half the length of the spore.

Germination.

A centre piece of wing, about 2 cm square, was carefully cut out and placed on a microscope slide. A small drop of 0.1% cotton blue in lactophenol was placed on the wing section and a coverslip placed on top. The wing piece was observed under the microscope. Counts were taken every 2 h from 6 to 12 h after inoculation, using a new wing piece each time. At each count five separate wing pieces were used, and on each piece 300 spores were counted, giving 1500 spores counted for each recording time. Individual spores and clumps of up to five spores were counted. Larger clumps were ignored as spores could not be clearly distinguished in clumps of greater than five.

Appressorium formation.

To assess appressorium formation a similar procedure was followed. Wings were sterilised in an atmosphere of propylene oxide and inoculated on 2% water agar plates as described above, then incubated at 27°C in high humidity. A 2 cm square centre section of wing was used, stained with 0.1% cotton blue in lactophenol. Counts were taken every 2 h for 8 h from the time appressoria were first noted, giving four observation times. At each count five separate wing pieces were used, and on each wing piece 10 fields of view were chosen, giving 50 readings per observation time. In each field of view the total number of spores were counted, and the total number of

appressoria, to give a value of the number of appressoria per spore. Large clumps of spores could not be clearly distinguished, therefore only single spores and clumps of up to five spores were counted. An appressorium was defined as an apical swelling on the germ tube, of a similar size to that of the germinated spore.

Penetration.

To assess penetration locust wings were inoculated and incubated in the same manner as for appressorium formation. A 2 cm square centre section of wing was used, stained with 0.1% cotton blue in lactophenol. The spores, germ tubes and appressoria stained blue, but the penetrant hyphae (between the upper and lower layers of the wing) were shielded from the stain and appeared transparent. Counts were taken every 2 h from the time penetrant hyphae were first noted and over the following 8 h, giving four observation times. At each count five separate wing pieces were used, and on each wing piece 10 fields of view were chosen, giving 50 readings per observation time. In each field of view the total number of spores were counted, and the total number of penetration points, to give a value of the number of penetration points per spore. Large clumps of spores could not be clearly distinguished, therefore only single spores and clumps of up to five spores were counted.

Enzyme assay.

The production of a number of proteases by 18 *Metarhizium anisopliae* and *flavoviride* isolates was assessed on excised locust wings. The wings were removed from adult locusts 3 to 7 days after ecdysis, surface sterilised overnight in the presence of propylene oxide and spread flat on water agar plates. The wings were inoculated with 150 µl of a 2.5 x 10^7 spores/ml spore solution. This was pipetted in small drops onto each wing, giving 3.75 x 10^6 spores/wing, six wings being inoculated per isolate. The wings were incubated at 27°C, until the time of initial penetration for that isolate (24 - 36 h, see Table 6).

Each wing was then cut up into pieces less than 0.5 cm^2 and shaken with 1 ml 50 mM N-(2-hydroxyethyl)piperazine-N'-(3-propane-sulfonic acid) (EPPS) buffer, pH8.0, at 4°C for 2 h. These enzyme extraction conditions were optimised in preliminary experiments using *M. anisopliae* isolate Me1. The enzyme extract was pipetted into a fresh tube, centrifuged at 13,000 rpm for 5 min in an MSE Micro Centaur and the supernatant removed and assayed.

Endoprotease PR1 was assayed by monitoring the release of nitroanilide at 405 nm from the substrate Suc-$(Ala)_2$-Pro-Phe-NA in a Dynatech MR5000 microtitre plate reader. 180 µl of the enzyme solution was pipetted into the microtitre plate, incubated at 27°C for 15 min before 20 µl of the substrate, dissolved in dimethyl formamide (DMF), was added. OD was determined every 6 sec for 5 min and the change in OD/min recorded as the enzyme activity.

PR2 and PR4 were assayed using Bz-Phe-Val-Arg-NA, as described for PR1. PR4 activity was defined as that activity vs. Bz-Phe-Val-Arg-NA which was not inhibitable by phenylmethylsulphonyl fluoride (PMSF). PMSF, dissolved in 20 µl of DMF, was added to the reaction mixture to give a final concentration of 1 mM for the PR4 assay. This was replaced with 20 µl of DMF alone for the PR2 + PR4 assay. After the addition of the inhibitor the plate was incubated for 30 min at 27°C before the reaction was started by the addition of the substrate. The reaction was followed as described for PR1.

These extraction and assay procedures were based on those used by St. Leger *et al.* (1987a and b). PR2 is a serine protease which is inhibited by PMSF (St. Leger *et al.*, 1987a). PR4 is a cysteine-like protease which is not inhibited by PMSF (Cole *et al.*, 1993). The substrates were used at a final concentration that would give Vmax., which for PR1 = 0.5 mM and for PR2 = 0.66 mM (St. Leger et. al., 1987a).

Statistics.

Chi-squared tests and T-tests were carried out on original data. Regression lines were fitted using Minitab by the least squares method.

RESULTS

Germination.

Initial germination experiments appeared to suggest a variation in germination rates, for a single isolate, between different wing regions. This was investigated further. A spore suspension of 10^7 spores/ml in 0.04% Tween 80 was sprayed on the wings. They were incubated at 27°C and after 5, 7, 9 and 11 h five wings were cut into 3 pieces (see Fig. 2) and the percentage germination of conidia determined for each piece. The data are shown in Table 2. Germination was first seen at 7 h and at this and each subsequent sampling period there was a consistent difference in percentage germination between regions in the order bottom>middle>top. At 11 h all three regions were significantly different from each other (Table 3).

There was no obvious reason why the different regions of the wing should promote germination to different extents. The middle region was then chosen as the region for assessment in the germination experiments with 19 *Metarhizium* isolates. This was because, although it did not give the greatest percentage germination, it was the clearest to collect data from, having less wing hairs than the bottom region, and taking the centre region would reduce any possible edge effects.

The values of mean, maximum and minimum percentage germination are give in Table 4. These values were plotted against time for each isolate and a selection of the graphs are shown in Fig. 3 (see Appendix II for graphs of all isolates). Many of the graphs are S-shaped, but regression lines have been fitted to the data to allow better analysis of the results, although it is recognised that this is a simplification of the data. Using the regression analysis a value for the rate of germination over the period of observation was obtained (Table 4). Seven of the isolates do not reach 50% germination by 12 h, and have the lowest rates of germination. The time of initial germination, calculated from the regression line, was between 6 and 8 h for all the isolates.

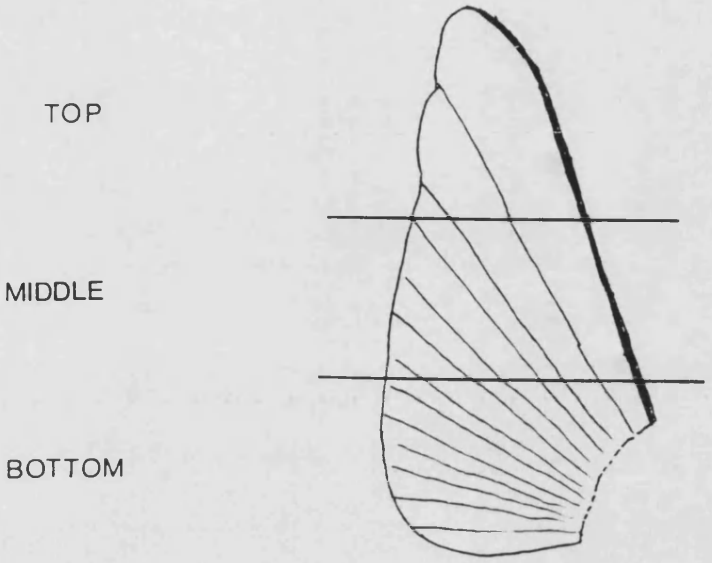


Figure 2. Diagrammatic representation of a fully extended locust hind wing, divided to show the three regions used in the germination experiments. Fold lines are shown. Not to scale.

Table 2. Percentage germination of *Metarhizium anisopliae* isolate Me1 spores on three different regions of the locust wing over time.

REGION	GERMINATION AT EACH TIME/hours			
	5	7	9	11
TOP	0	5.7	32.4	81.6
MIDDLE	0	5.1	40.1	86.9
BOTTOM	0	6.2	58.0	90.4

Table 3. Statistical comparison of germination after 11 hours on the three regions of the locust wing.

REGIONS	CHI-SQUARED VALUE	SIGNIFICANCE
TOP & MIDDLE	$X^2 = 13.75$ at $p = 0.05$	SIG
TOP & BOTTOM	$X^2 = 41.27$ at $p = 0.05$	SIG
MIDDLE & BOTTOM	$X^2 = 7.66$ at $p = 0.05$	SIG

Table 4. Mean, maximum and minimum percentage germination values and the rate of germination on locust wings, for the nineteen *Metarhizium anisopliae* and *flavoviride* isolates studied.

ISOLATE	% GERMINATION AT TIMES POST INOCULATION (MAX/MIN)				RATE OF GERM^{N} *
	6h	8h	10h	12h	
Arsef 324	0.84 (1.28/0.32)	22.65 (33.55/14.56)	69.20 (80.25/56.05)	84.99 (92.65/65.69)	14.95
324673	3.28 (6.93/0.64)	13.25 (24.33/8.70)	55.80 (66.34/42.21)	83.17 (93.51/66.33)	14.11
299984	4.60 (7.33/1.00)	48.07 (62.00/22.67)	83.47 (88.33/77.67)	83.26 (93.33/73.33)	13.57
298059	0 (0/0)	0.33 (1.33/0)	1.67 (4.00/0.33)	16.07 (26.00/10.00)	2.48
299981	5.13 (13.33/0)	25.47 (44.67/15.33)	60.13 (77.67/50.00)	88.80 (96.00/80.00)	14.28
168777ii	7.66 (16.17/2.60)	40.67 (68.49/26.14)	49.90 (68.06/27.67)	64.61 (89.70/31.23)	9.00
152222	0 (0/0)	0.07 (0.33/0)	1.54 (2.82/1.00)	20.75 (26.71/14.29)	3.19
Arsef 2023	0.40 (1.32/0)	9.48 (21.40/0)	55.21 (66.00/44.70)	74.75 (85.15/66.34)	13.44
I91 676	20.13 (40.00/6.67)	53.73 (64.33/44.33)	70.33 (77.67/60.67)	79.33 (85.67/65.33)	9.71
Arsef 727	1.60 (3.33/0)	23.67 (31.33/11.67)	50.60 (78.33/30.00)	64.47 (78.00/51.67)	10.78
Arsef 438	0.53 (1.33/0)	2.87 (7.67/0)	15.20 (23.33/7.00)	28.47 (47.67/14.67)	4.81
Arsef 440	0 (0/0)	0.33 (1.67/0)	0.27 (1.00/0)	2.13 (5.67/0)	0.32
Arsef 439	0 (0/0)	0.47 (1.00/0)	3.20 (6.00/0.33)	16.93 (23.33/10.00)	2.68
298061	41.13 (54.33/30.33)	79.93 (90.33/69.67)	90.80 (96.33/78.67)	95.00 (97.67/91.33)	8.62
Me 1	48.55 (74.48/15.69)	73.28 (80.92/57.86)	85.43 (95.10/78.90)	97.90 (99.34/96.33)	8.01
Nr 48	0.26 (0.99/0)	6.71 (15.84/1.66)	12.22 (19.54/4.21)	41.45 (76.95/16.61)	6.45
330189	1.43 (2.21/0.66)	23.25 (39.82/5.50)	69.02 (83.17/51.45)	88.63 (94.57/80.00)	15.37
I90 574	15.60 (21.30/12.00)	65.00 (87.70/41.30)	70.90 (91.30/55.70)	73.20 (81.30/66.30)	8.94
I91 633	0 (0/0)	0 (0/0)	1.90 (3.70/0.30)	13.30 (23.30/7.30)	2.09

* Rate of germination = percentage germination per hour

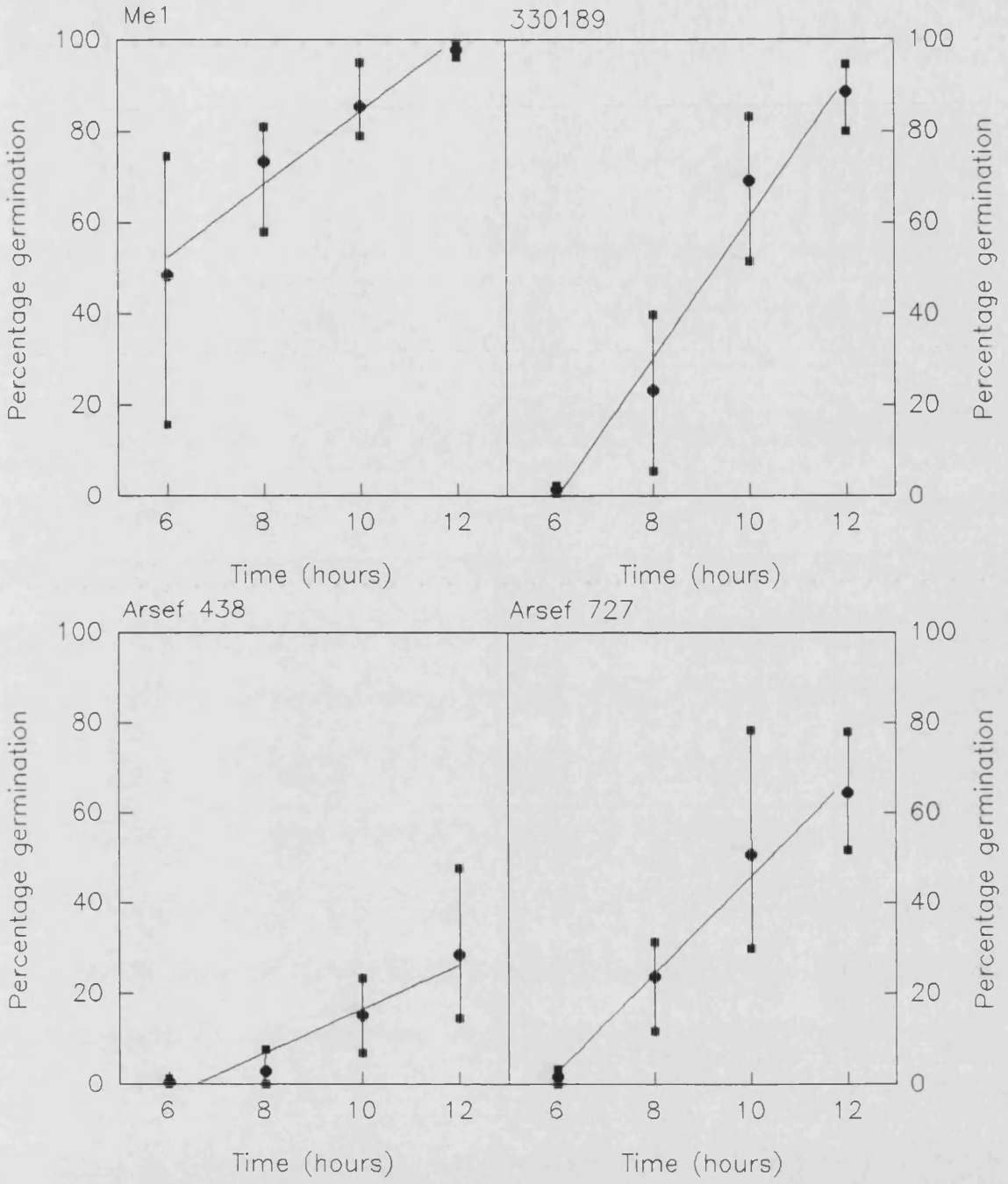


Figure 3. Mean, maximum and minimum percentage germination of four Metarhizium isolates on excised locust wings, incubated at 27°C for 6, 8, 10 and 12 h post inoculation. Regression lines have been fitted to the data.

Appressorium formation.

Appressoria were produced on germ tubes as early as 9 h after inoculation for some isolates but not until 28 h after inoculation for others. They appeared as apical swellings on the germ tubes, of a similar size to the swollen, germinated conidia, but were easily distinguished from the conidia as many were curved and longer than the conidia. The mean number of appressoria per spore (shown in Table 5) was plotted against time for each isolate. Regression lines were fitted to the data and from these both the time of initial formation and the rate of formation of appressoria were calculated (see Table 5). A selection of these graphs are shown in Fig. 4 (see Appendix II for graphs of all isolates).

The length of the germ tube the spore produces before forming an appressorium is seen to vary. In Fig. 5 the appressoria of the isolate I90 574 are being produced on germ tubes only 3-5 times the length of the spore. In Fig. 6 the germ tubes of Me1 spores are about 10 times the spore length when the appressoria are produced. In both of these Figs. the germ tubes are relatively short and either unbranched or just beginning to branch. Figs. 7 and 8 show much longer germ tubes of isolates Arsef 440 and Arsef 438 that are highly branched. Appressoria formed on each branch. In all isolates germ tube branching and more than one germ tube per spore were seen at some stage during the prepenetration process. All isolates also eventually had long branched hyphae with appressoria on many branches, such as in Fig. 7. Any differences between isolates that were observed were in the initial stages of appressoria formation. Generally, the more pathogenic isolates (see later) produced only short (<10 times spore length) germ tubes before an appressorium was formed. The less pathogenic isolates tended to produce long, branched hyphae and more than one germ tube , as in Fig. 8, before any appressoria were formed.

M. flavoviride isolate Arsef 2023 was found not to produce appressoria on the wing until many hours after the other isolates. Instead, Arsef 2023 appeared to produce spores. In the centre of Fig. 9 can be seen a germinated spore that has produced a single germ tube, on the end of which three spores have formed. Fig. 10 shows many branched

Table 5. Number of appressoria per spore for eighteen *Metarhizium anisopliae* and *flavoviride* isolates, every 2 h from the time of initial appressoria observation (time of first count). The rate of appressoria formation and the time of initial appressoria formation were calculated from a regression line drawn through the data.

ISOLATE	TIME 1st COUNT -h	No. APPRESSORIA/SPORE EVERY 2h FROM TIME OF 1st COUNT Mean (SE)				RATE * FORMn	INITIAL TIME -h
Arsef 324	20	0.025 (0.012)	0.041 (0.013)	0.154 (0.028)	0.208 (0.028)	0.033	20
324673	20	0.095 (0.023)	0.095 (0.018)	0.133 (0.019)	0.257 (0.041)	0.026	18
299984	28	0.022 (0.008)	0.026 (0.007)	0.022 (0.006)	0.053 (0.018)	0.004	24
298059	20	0.067 (0.020)	0.165 (0.030)	0.251 (0.047)	0.400 (0.051)	0.054	19
299981	12	0.018 (0.006)	0.126 (0.026)	0.165 (0.026)	0.264 (0.034)	0.039	11
168777ii	10	0.027 (0.011)	0.052 (0.013)	0.194 (0.033)	0.169 (0.025)	0.029	9
152222	26	0.092 (0.020)	0.125 (0.024)	0.145 (0.024)	0.241 (0.041)	0.023	23
I91 676	10	0.018 (0.006)	0.058 (0.015)	0.074 (0.020)	0.158 (0.022)	0.022	9
Arsef 727	26	0.285 (0.037)	0.500 (0.047)	0.493 (0.045)	0.759 (0.053)	0.071	22
Arsef 438	30	0.105 (0.003)	0.162 (0.031)	0.167 (0.031)	0.364 (0.047)	0.039	28
Arsef 440	28	0.117 (0.022)	0.154 (0.022)	0.214 (0.031)	0.252 (0.035)	0.023	23
Arsef 439	28	0.058 (0.013)	0.119 (0.020)	0.098 (0.018)	0.114 (0.020)	0.007	18
298061	10	0.002 (0.002)	0.056 (0.015)	0.084 (0.021)	0.150 (0.026)	0.024	10
Mel	12	0.021 (0.006)	0.064 (0.012)	0.102 (0.021)	0.170 (0.028)	0.024	11
Nr 48	26	0.102 (0.023)	0.161 (0.029)	0.199 (0.037)	0.262 (0.044)	0.026	22
330189	26	0.121 (0.027)	0.093 (0.031)	0.116 (0.027)	0.428 (0.051)	0.047	25
I90 574	16	0.157 (0.029)	0.210 (0.034)	0.316 (0.041)	0.532 (0.042)	0.062	14
I91 633	30	0.041 (0.009)	0.073 (0.019)	0.125 (0.023)	0.093 (0.019)	0.010	25

*Rate of appressoria formation = No. appressoria per spore per hour

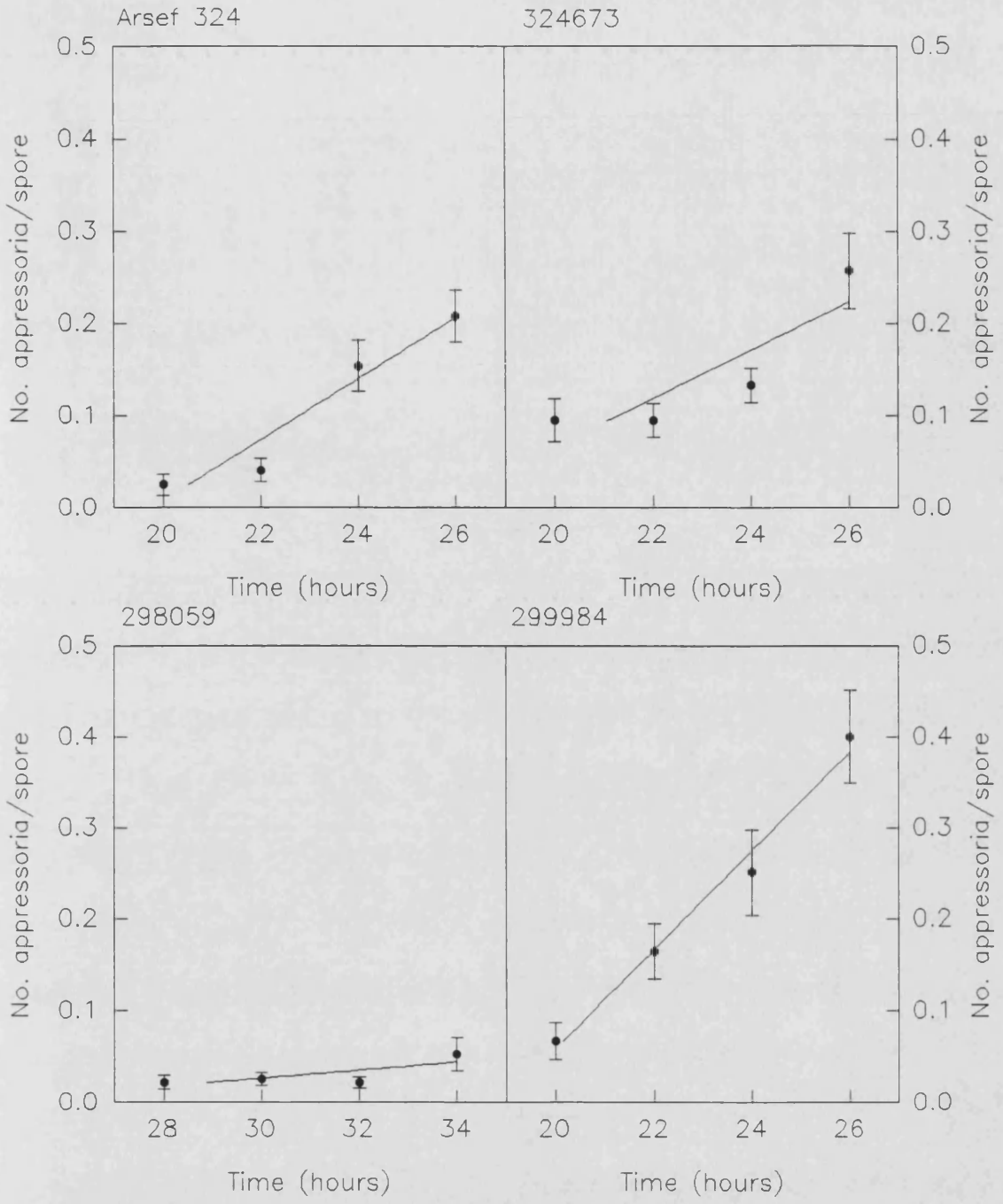


Figure 4. Number of appressoria/spore (mean ± SE) for three Metarhizium anisopliae and one M. flavoviride isolates on excised locust wings, incubated at 27°C and observed over a six hour period from the time of initial appressoria formation. Regression lines have been fitted to the data.

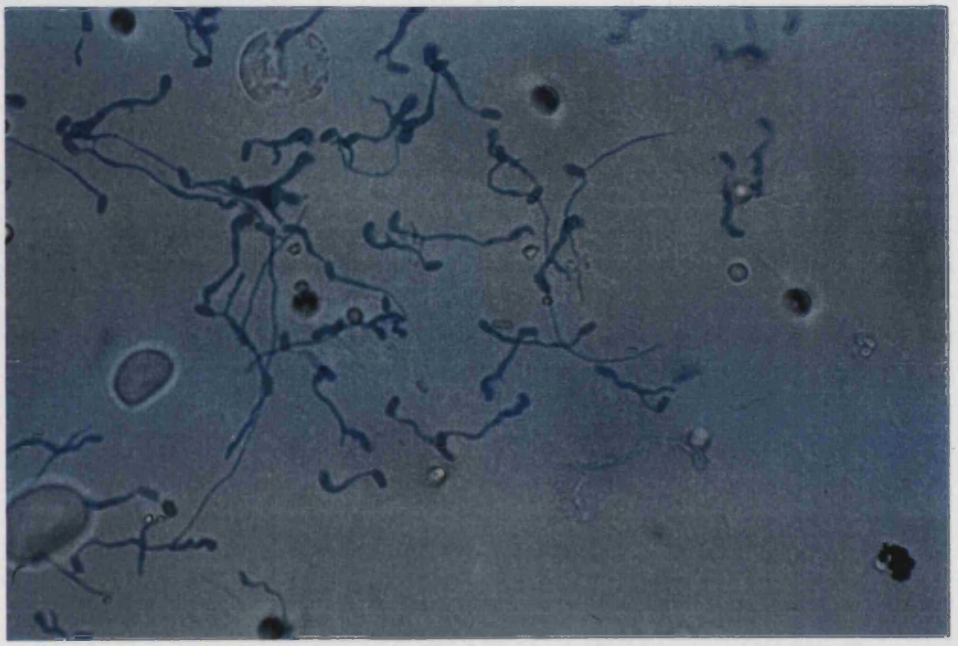


Figure 5. Germinated spores of *Metarhizium anisopliae* isolate I90 574 with appressoria formed on relatively short germ tubes, many only 3 - 5 times the length of the spore.

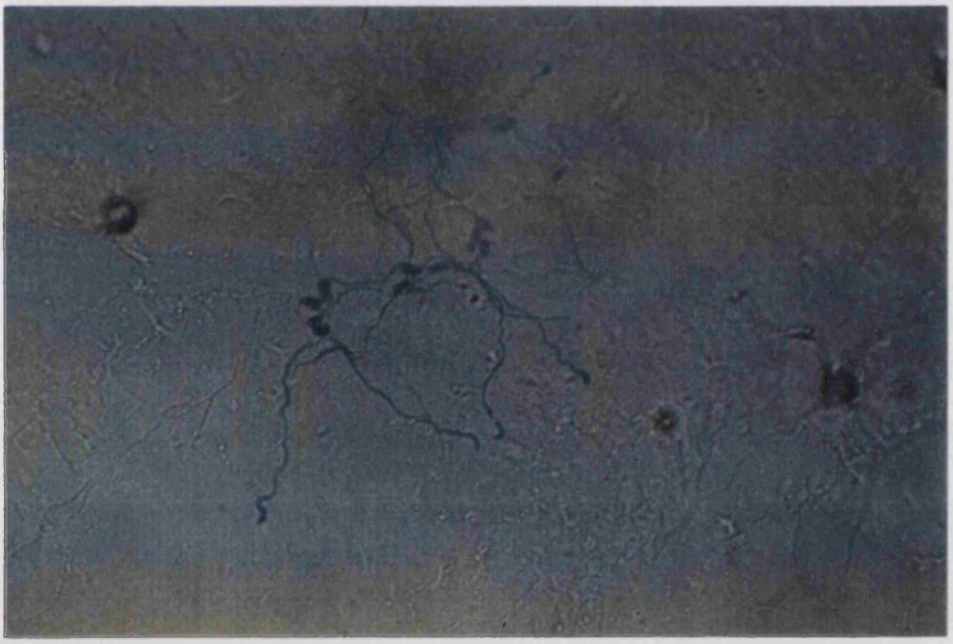


Figure 6. Germinated spores of *Metarhizium anisopliae* isolate Me1. The germ tubes are about 10 times the length of the spore before appressoria are produced.

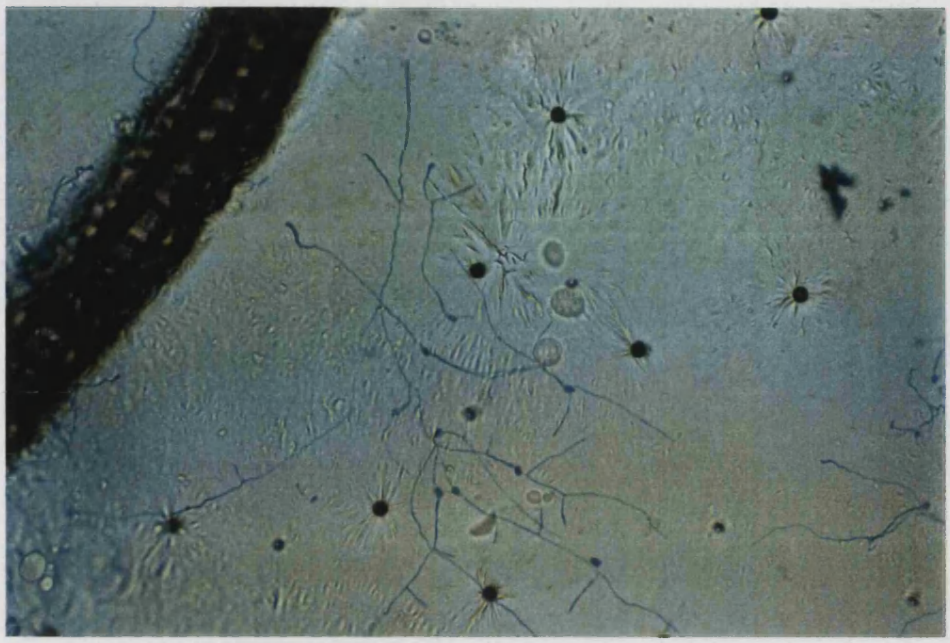


Figure 7. Germinated spores of *Metarhizium anisopliae* isolate Arsef 440 with long branched germ tubes and appressoria forming at the tips.

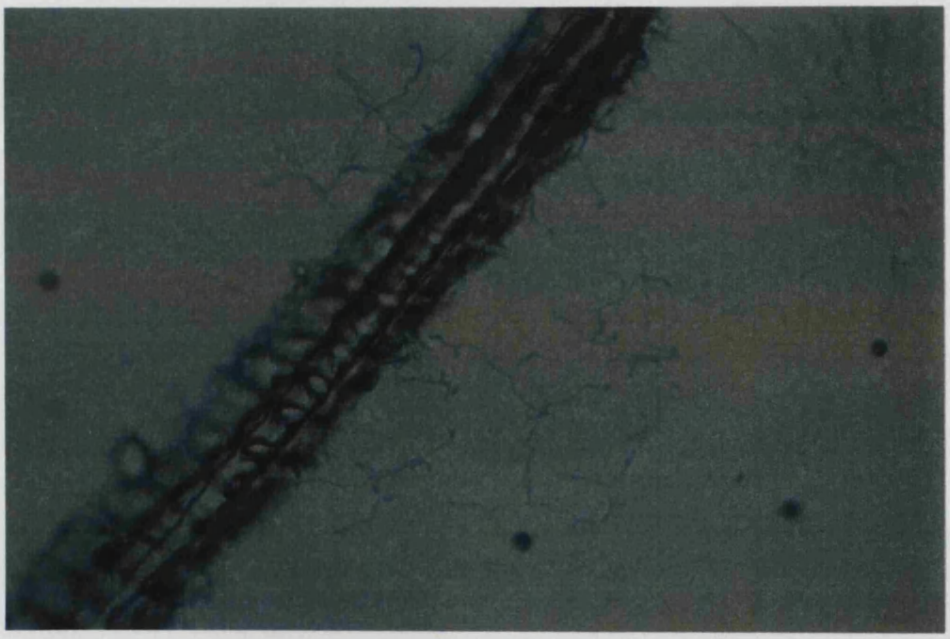


Figure 8. Germinated spores of *Metarhizium anisopliae* isolate Arsef 438 with appressoria forming on long branched hyphae.

Figure 9. *Metarhizium flavoviride* isolate Arsef 2023. The spore in the centre of the photograph can be seen to have germinated and produced a short germ tube on which 3 spores have developed.

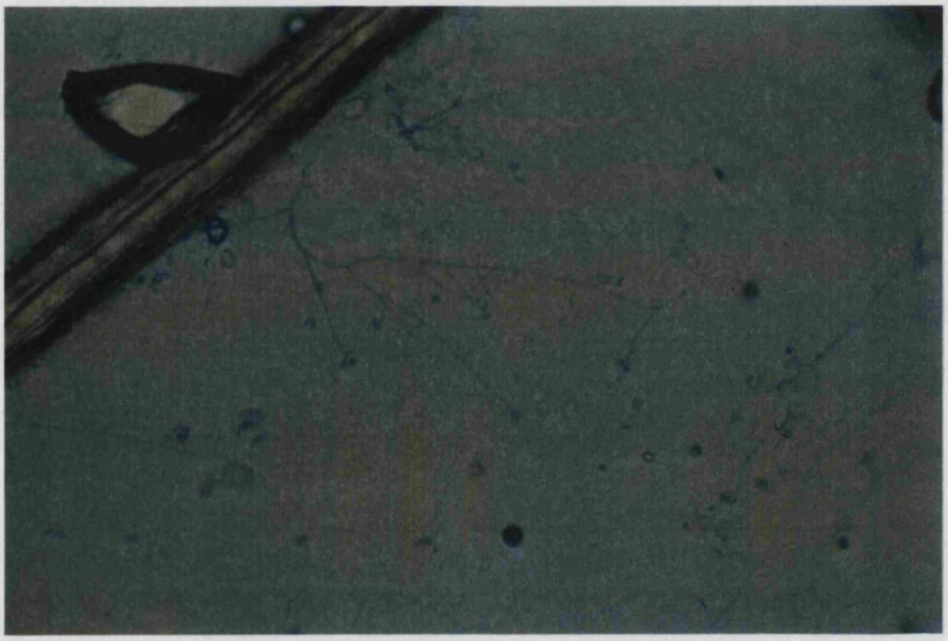


Figure 10. A later stage in the growth of *Metarhizium flavoviride* isolate Arsef 2023. There is extensive hyphal branching, with clumps of up to 4 spores developing on the tips of the hyphal branches.

hyphae with clumps of up to 4 spores at the tips of branches, but no appressoria are apparent. This spore production on the wing meant that no values could be obtained for the number of appressoria/spore or penetration points/spore for this isolate because there were many more spores present than those originally sprayed onto the wing.

Penetration.

Penetration was seen to occur from 24 to 36 h after inoculation. Penetration pegs and/or penetrant hyphae grew down into the wing from the appressoria. Figs. 11 and 12 show penetration of the locust wing by unstained penetrant hyphae and growth of those penetrant hyphae (unstained) between the upper and lower cuticular surfaces of the wing, as compared with the blue stained germ tubes and spores on top of the wing. In Fig. 11 a single penetrant hypha is seen to be growing into the wing, from a single germinated spore above the wing. Fig. 12 shows penetration from a clump of spores, and, although clumps of spores were not counted, it can be seen that mass penetration did occur from clumps of spores.

The mean number of penetration points per spore (Table 6) was plotted against time for each isolate. A regression line was drawn through the data and a value of the rate of penetration and the time of initial penetration were calculated (see Table 6). A selection of these plots are shown in Fig. 13 (see Appendix II for plots of all isolates).

The two remaining *M. flavoviride* isolates, 330189 and 324673, behaved like Arsef 2023, producing spores on the wing. These isolates germinated normally and produced appressoria without spore production on the wing in previous experiments. The reason for this change in growth form was not obvious. The experiment was repeated on fresh cultures of 330189 and 324673 with similar results. Although penetration was seen and the time of initial penetration was calculated from observation of the isolates no counts of the number of penetration points/spore could be obtained because of the spores produced on the wing.

Figure 11. Penetration of the locust wing by a penetrant hypha (clear) from a germinated spore and appressorium (stained blue).

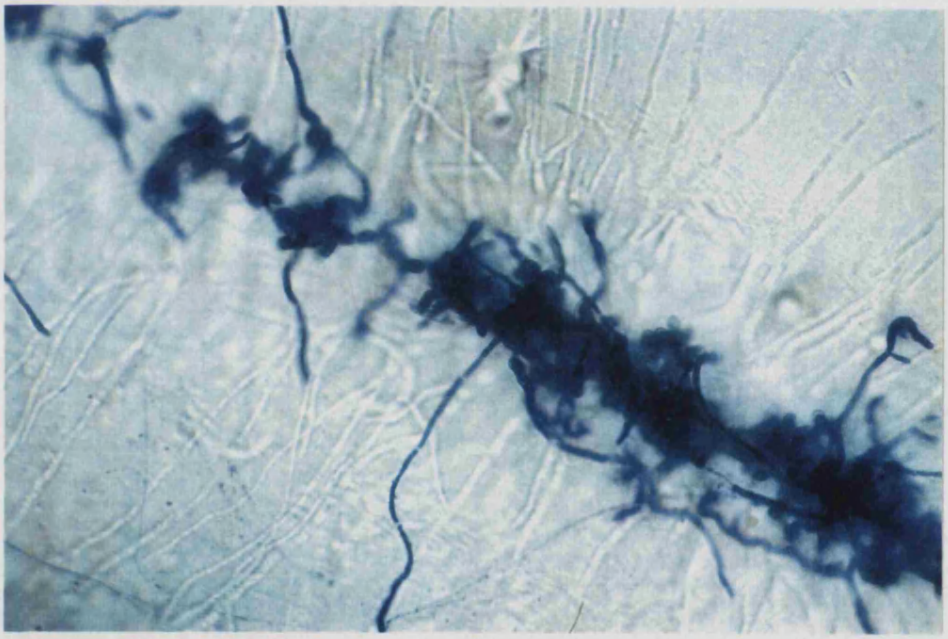


Figure 12. Multiple penetration of the locust wing, with many clear penetrant hyphae growing out within the wing, from a large clump of spores on the surface.

Table 6. Number of penetration points per spore for eighteen *Metarhizium anisopliae* and *flavoviride* isolates, for every 2 h from the time of initial penetration (time of first count, calculated from preliminary experiments). The rate of penetration and the time of initial penetration were calculated from a regression line drawn through the data.

ISOLATE	TIME 1st COUNT-h	No. PENETRATION POINTS/SPORE EVERY 2h FROM TIME OF FIRST COUNT Mean (SE)				RATE OF PENET^N *	INITIAL PENET^N
Arsef 324	26	0 (0)	0.003 (0.002)	0.025 (0.008)	0.062 (0.015)	0.0104	28
299984	32	0.001 (0.001)	0.004 (0.002)	0.006 (0.003)	0.016 (0.006)	0.0023	32
298059	34	0.052 (0.013)	0.157 (0.034)	0.130 (0.027)	0.221 (0.026)	0.0246	32
299981	24	0.005 (0.003)	0.002 (0.001)	0.007 (0.003)	0.036 (0.011)	0.0051	24
168777ii	24	0.008 (0.007)	0.044 (0.016)	0.064 (0.017)	0.097 (0.020)	0.0144	24
152222	32	0.014 (0.004)	0.011 (0.005)	0.009 (0.004)	0.015 (0.006)	0.0001	32
I91 676	24	0.008 (0.005)	0.097 (0.022)	0.045 (0.020)	0.158 (0.025)	0.0199	24
Arsef 727	26	0.011 (0.005)	0.033 (0.011)	0.085 (0.017)	0.157 (0.024)	0.0245	26
Arsef 438	34	0.026 (0.012)	0.056 (0.017)	0.048 (0.021)	0.078 (0.020)	0.0075	32
Arsef 440	32	0.003 (0.002)	0 (0)	0.003 (0.002)	0.004 (0.003)	0.0003	32
Arsef 439	36	0.016 (0.004)	0.040 (0.014)	0.015 (0.005)	0.022 (0.007)	0	32
298061	26	0.122 (0.022)	0.117 (0.025)	0.349 (0.042)	0.355 (0.038)	0.0466	24
Me1	24	0.011 (0.008)	0.033 (0.010)	0.047 (0.012)	0.091 (0.020)	0.0127	24
Nr 48	34	0.006 (0.003)	0.004 (0.002)	0.016 (0.010)	0.013 (0.005)	0.0017	34
I90 574	24	0.001 (0.001)	0.022 (0.008)	0.043 (0.013)	0.062 (0.015)	0.0102	24
I91 633	36	0.006 (0.003)	0.012 (0.005)	0.005 (0.003)	0.026 (0.007)	0.0026	36

* Rate of penetration = No. penetration points per spore per hour

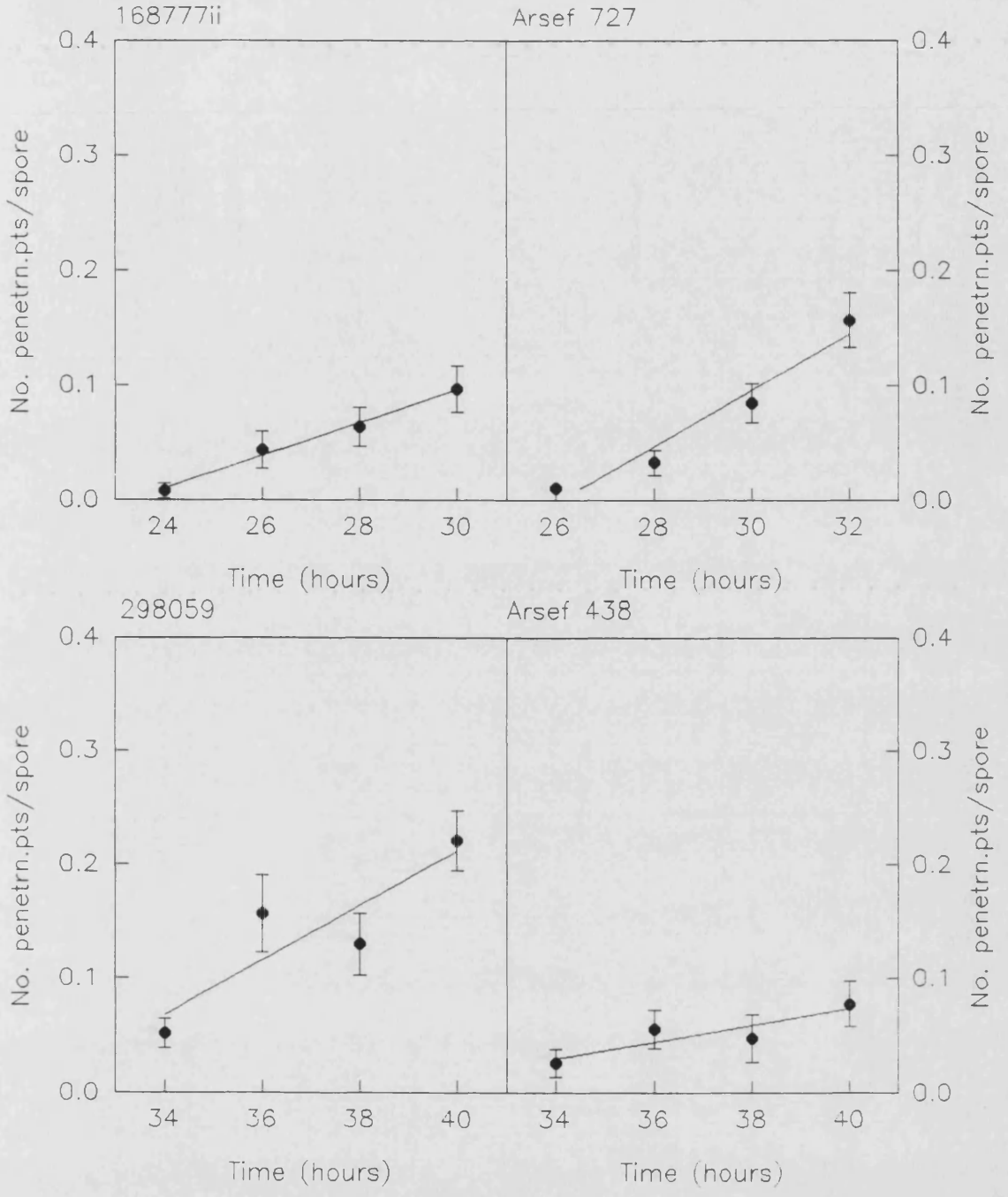


Figure 13. Number of penetration points/spore (mean ± SE) for four Metarhizium anisopliae isolates on excised locust wings, incubated at 27°C and observed over a six hour period from the time of initial penetration. Regression lines have been fitted to the data.

Enzyme assays.

The levels of enzyme activity against the two protease substrates for each of 18 isolates are given in Table 7 and plotted in Figs. 14 and 15. Protease PR1 activity against Suc-$(Ala)_2$-Pro-Phe-NA for 14 of the 18 isolates is seen to be at a very low level (Fig. 14). 168777ii, Arsef 439, Me1 and Arsef 438 have higher levels, with Arsef 438 producing considerably more PR1 than any of the other isolates.

Total activity against the Bz-Phe-Val-Arg-NA substrate (=PR2 plus PR4 activity) follows a similar pattern. Most of the isolates have low levels of PR2+PR4 activity, with only Arsef 438 and Me1 producing more substantial amounts (Fig. 15).

In the presence of PMSF the level of activity against the Bz-Phe-Val-Arg-NA substrate for Me1 is halved, indicating that PR2 accounts for half of the activity versus this substrate. This would appear to be the case for a number of the other isolates also, but some (Arsef 438, Arsef 439, and Arsef 440) are seen to have higher proportions of PR4.

A second inhibitor, 5-5'-dithiobis-(2 nitrobenzoic acid) (DTNB), an inhibitor of PR4 and other cysteine proteases (Cole *et al.*, 1993) (used like PMSF at a final concentration of 1 mM) was used in initial experiments, but controls were very high due to the yellow colour of the inhibitor. This lead to inhibition only being apparent with high levels of enzyme activity, such as with Me1. In initial experiments with Me1 the mean level of activity in the presence of the Bz-Phe-Val-Arg-NA substrate alone (therefore for PR2+PR4) was 0.0837 OD/min (±0.0042). With PMSF present the activity (for PR4) was 0.0342 OD/min (±0.0020) and with DTNB present the activity (for PR2) was 0.0540 OD/min (±0.0041). This would indicate that for Me1 the PR2 and PR4 account for the total activity against this substrate.

Correlations between parameters and isolate virulence.

An attempt was made to correlate germination, appressorium formation, penetration and enzyme experiments with isolate virulence for the 19 *Metarhizium* isolates studied. Virulence was expressed as median lethal time (MLT) of the isolates

Table 7. Change in OD/min for eighteen *Metarhizium anisopliae* and *flavoviride* isolates with two separate protease substrates. The PR1 column is the activity against the Suc-$(Ala)_2$-Pro-Phe-NA substrate alone, the PR2 + PR4 column is the activity against the Bz-Phe-Val-Arg-NA substrate alone and the PR4 column is the activity against the Bz-Phe-Val-Arg-NA substrate in the presence of the inhibitor PMSF. All activities are adjusted for the control. Mean (S.E.) are given. n = 6.

ISOLATE	**PR1 OD/min**	**PR2 + PR4 OD/min**	**PR4 OD/min**
Arsef 324	0.0262 (0.0032)	0.0064 (0.0009)	0.0026 (0.0005)
324673	0.0012 (0.0007)	0.0022 (0.0012)	0.0017 (0.0009)
299984	0.0018 (0.0006)	0.0035 (0.0009)	0.0032 (0.0009)
298059	0.0078 (0.0040)	0.0020 (0.0008)	0.0011 (0.0005)
299981	0.0068 (0.0015)	0.0037 (0.0012)	0.0014 (0.0006)
168777ii	0.0573 (0.0099)	0.0065 (0.0018)	0.0022 (0.0009)
152222	0.0048 (0.0016)	0.0024 (0.0008)	0.0029 (0.0007)
I91 676	0.0148 (0.0036)	0.0180 (0.0036)	0.0082 (0.0017)
Arsef 727	0.0088 (0.0018)	0.0107 (0.0026)	0.0066 (0.0017)
Arsef 438	0.2493 (0.0170)	0.0420 (0.0048)	0.0351 (0.0052)
Arsef 440	0.0005 (0.0004)	0.0150 (0.0026)	0.0121 (0.0020)
Arsef 439	0.0867 (0.0189)	0.0107 (0.0049)	0.0087 (0.0035)
298061	0.0100 (0.0043)	0.0142 (0.0027)	0.0049 (0.0012)
Mel	0.1306 (0.0083)	0.0546 (0.0054)	0.0288 (0.0026)
Nr48	0.0020 (0.0009)	0.0020 (0.0014)	0.0006 (0.0005)
330189	0.0000 (0.0007)	0.0095 (0.0011)	0.0026 (0.0012)
I90 574	0.0010 (0.0002)	0.0035 (0.0015)	0.0009 (0.0006)
I91 633	0.0003 (0.0003)	0.0012 (0.0010)	0.0002 (0.0009)

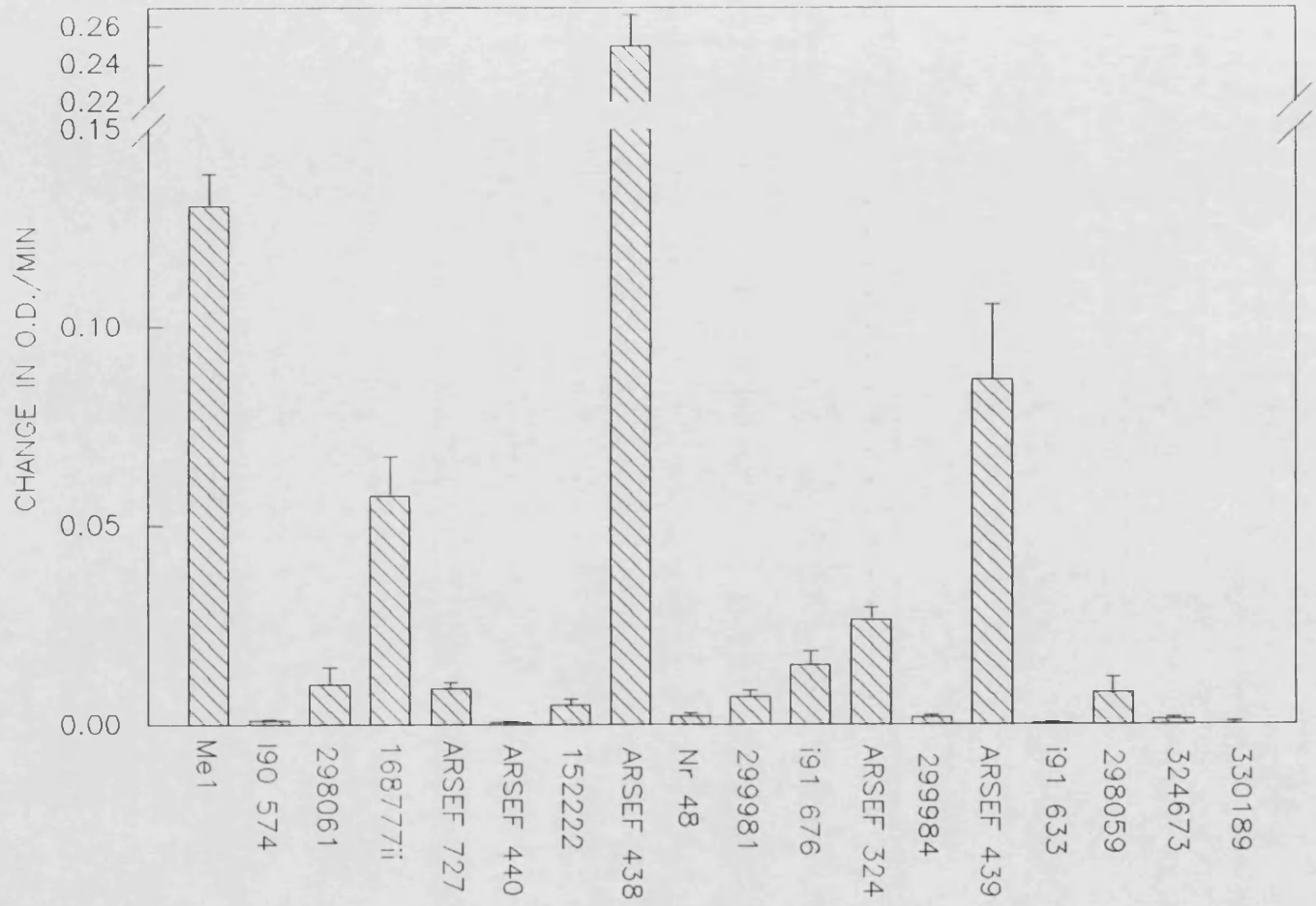


FIG. 14. PR1 activity for eighteen Metarhizium anisopliae and flavoviride isolates against the protease substrate Suc–$(Ala)_2$–Pro–Phe–NA. Activities have been adjusted for the control. Mean ± SE. 6 replicates for each isolate.

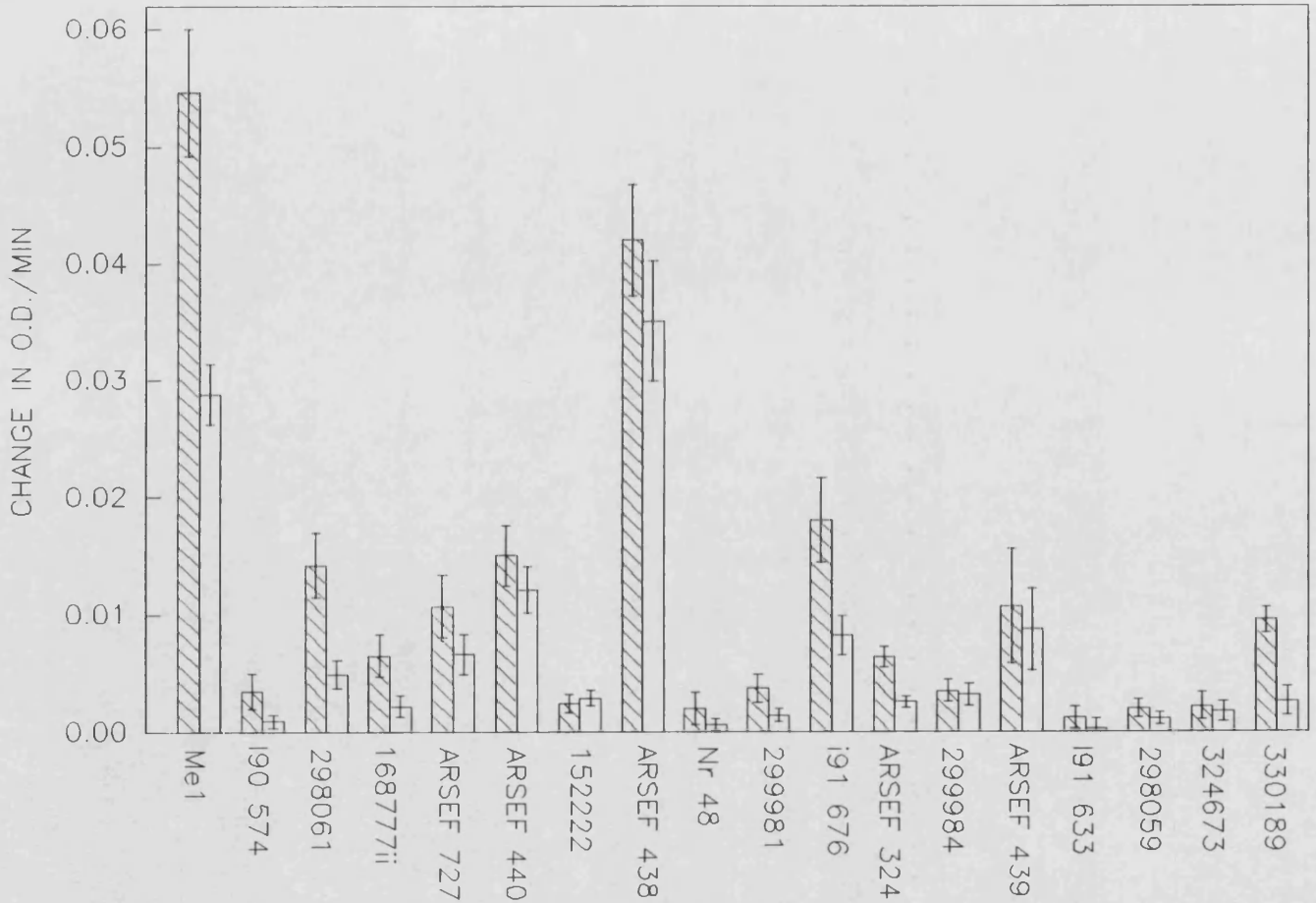


Figure 15. Protease activity for eighteen Metarhizium anisopliae and flavoviride isolates against the protease substrate Bz–Phe–Val–Arg–NA.
▨ = activity against substrate alone, therefore PR2+PR4 activity.
□ = activity against substrate plus inhbitor, PMSF, therefore PR4 activity.
Activities have been adjusted for the control. Mean ± SE.
6 replicates for each isolate.

for adult desert locusts, *S. gregaria*. The bioassay data was supplied by IIBC (see Appendix III). The parameters in Tables 8 and 9 were also plotted individually against MLT (see Figs. 16 and 17).

Using Minitab a correlation coefficient was calculated for each parameter vs. MLT. The correlation coefficients are given in Table 10. A significant negative correlation was found between MLT and the rate of germination (correlation coefficient = -0.554, $p = 0.05$). No other significant correlations occurred.

The time of initial germination varied by as little as 2 h (6 to 8 h post inoculation) and half of the isolates had the same time of initial penetration therefore neither of these parameters could be used to correlate with MLT.

Table 8. Median lethal time (M.L.T.) against the desert locust for 19 *Metarhizium anisopliae* and *flavoviride* isolates, and the rate of germination, the time of initial appressoria formation, the rate of appressoria formation and the rate of penetration for the same isolates on excised locust wings. (See text for further details on units of measurement for each parameter.)

ISOLATE	**M.L.T.**	**Germination rate**	**Initial time of appressoria formation**	**Rate of appressoria formation**	**Rate of penetration**
Arsef 324	3.71	15	20	0.033	0.0104
330189	4.41	15	25	0.047	-
324673	4.59	14	18	0.026	-
Me1	4.82	8	11	0.024	0.0127
Nr48	4.83	6	22	0.026	0.0017
168777ii	4.96	9	9	0.029	0.0144
Arsef 2023	5.39	13	-	-	-
152222	5.48	3	23	0.023	0.0001
I91 676	6.05	10	9	0.022	0.0199
298061	7.42	9	10	0.024	0.0466
298059	8.08	5	19	0.054	0.0246
299984	9.03	14	24	0.004	0.0023
299981	9.47	14	11	0.039	0.0051
Arsef 727	10.5	11	22	0.071	0.0245
I90 574	11.12	9	14	0.062	0.0102
Arsef 438	13 *	5	28	0.039	0.0075
I91 633	13 *	2	25	0.010	0.0026
Arsef 440	13 *	0.3	23	0.023	0.0003
Arsef 439	13 *	3	18	0.007	0.0000

* - As these isolates had not reached 50% mortality by day 12 their MLT values are greater than 12 and therefore they were allotted a value of 13.

Table 9. Median lethal time (M.L.T.) against the desert locust for 18 *Metarhizium anisopliae* and *flavoviride* isolates, and the activity of proteases produced by those isolates on excised locust wings. (See text for further details on the measurement of enzyme levels.)

Isolates	**M.L.T.**	**PR1**	**PR2 + PR4**	**PR4**	**PR2**	**Ratio PR1:PR2+4**	**Ratio PR2:PR4**
Arsef 324	3.71	0.0262	0.0064	0.0026	0.0038	4.09	1.46
330189	4.41	0.00	0.0095	0.0026	0.0069	0	2.65
324673	4.59	0.0012	0.0022	0.0017	0.0005	0.55	3.4
Me1	4.82	0.1306	0.0546	0.0288	0.0258	2.39	0.88
Nr48	4.83	0.0020	0.0020	0.0006	0.0014	1.0	2.33
168777ii	4.96	0.0573	0.0065	0.0022	0.0043	8.82	1.95
152222	5.48	0.0048	0.0024	0.0029	0.00	2.00	0
I91 676	6.05	0.0148	0.0180	0.0082	0.0098	0.82	1.20
298061	7.42	0.0100	0.0142	0.0049	0.0093	0.70	1.90
298059	8.08	0.0078	0.0020	0.0011	0.0009	3.90	0.82
299984	9.03	0.0018	0.0035	0.0032	0.0003	0.51	0.09
299981	9.47	0.0068	0.0037	0.0014	0.0023	1.84	1.64
Arsef 727	10.5	0.0088	0.0107	0.0066	0.0041	0.82	0.62
I90 574	11.12	0.0010	0.0035	0.0009	0.0026	0.29	2.89
I91 633	13 *	0.0003	0.0012	0.0002	0.0010	0.25	5.00
Arsef 438	13 *	0.2493	0.0420	0.0351	0.0069	5.94	0.20
Arsef 439	13 *	0.0867	0.0107	0.0087	0.0020	8.10	0.23
Arsef 440	13 *	0.0005	0.0150	0.0121	0.0029	0.03	0.24

* - As these isolates had not reached 50% mortality by day 12 their MLT values are greater than 12 and therefore they were allotted a value of 13.

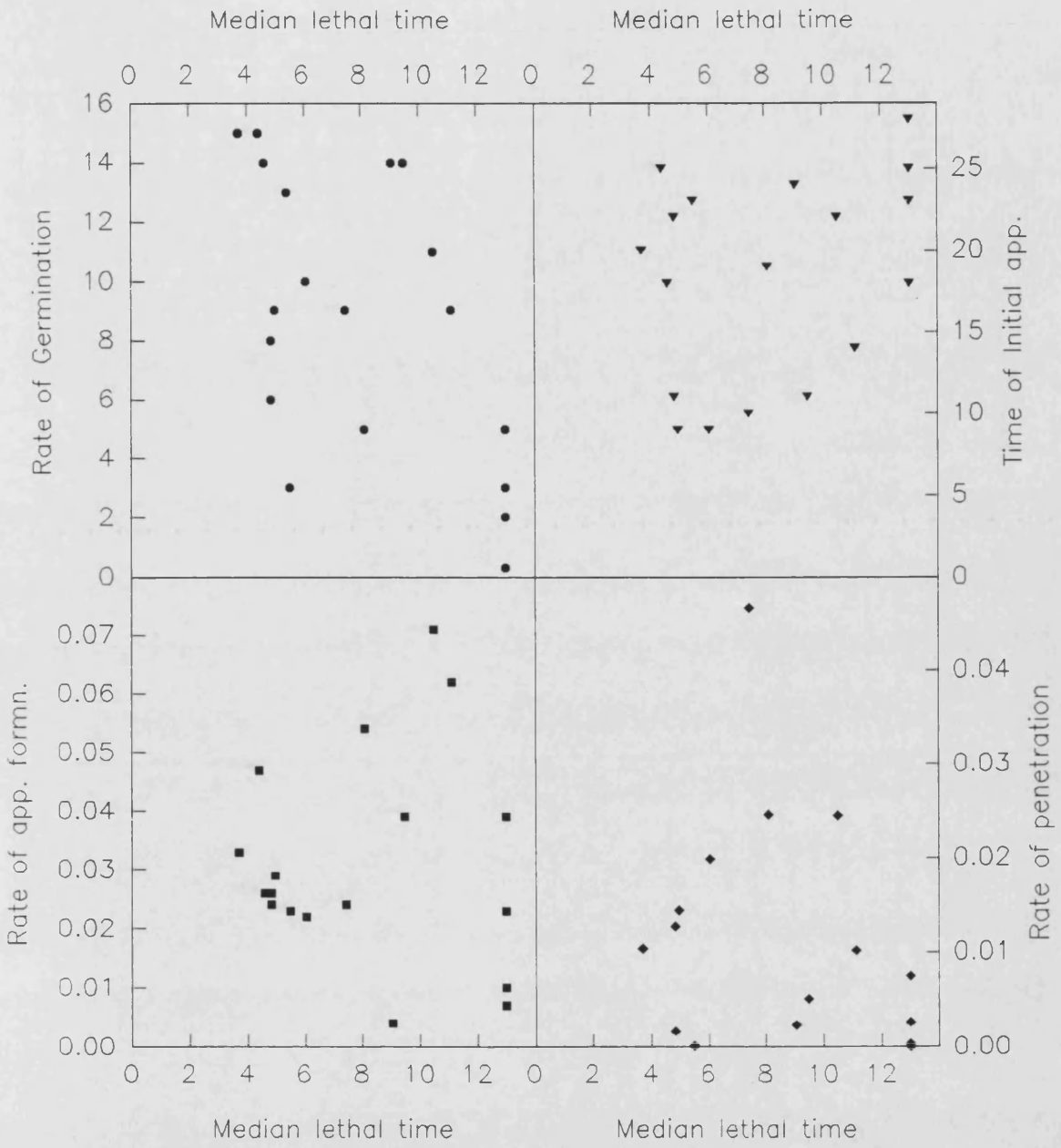


Figure 16. Plots of median lethal time against the rate of germination (•), the time of initial appressoria formation (▾), the rate of appressoria formation (▪) and the rate of penetration (◆).

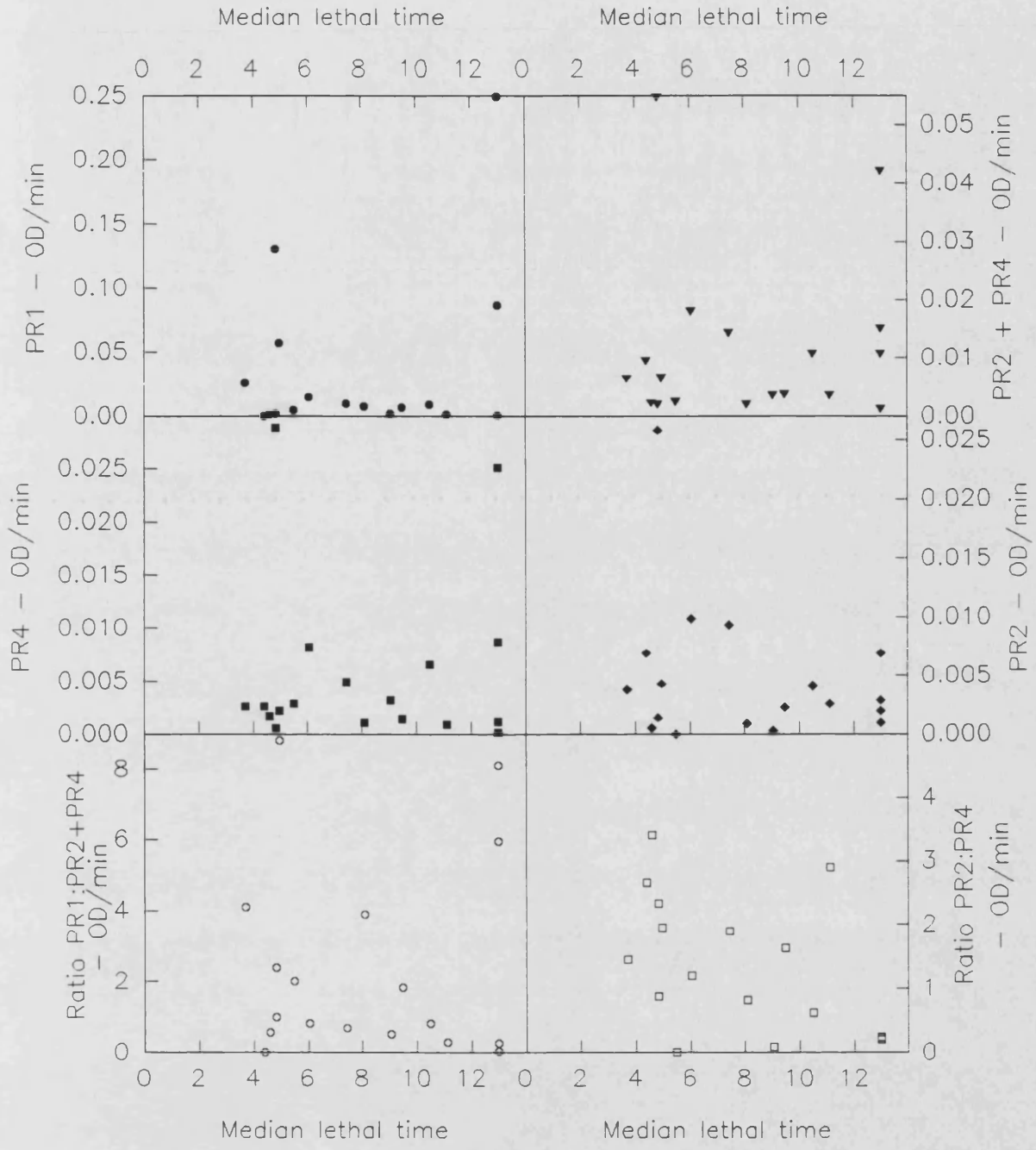


Figure 17. Plots of median lethal time against protease activity.
• = PR1, ▾ = PR2 + PR4,
▪ = PR4, ◆ = PR2,
○ = ratio PR1:PR2+PR4, ▫ = ratio PR2:PR4.

Table 10. Correlation coefficients for ten potential virulence determinants of eighteen *Metarhizium anisopliae* and *flavoviride* isolates when correlated with median lethal time (MLT) of those isolates. See text for details of the virulence determinants and median lethal time.

VIRULENCE DETERMINANTS	**CORRELATION COEFFICIENT**	**DEGREES OF FREEDOM**	**SIGNIFICANCE/ P VALUE**
Rate of germination	-0.554	17	SIG / p=0.05
Time of initial appressoria formation	0.317	15	NOT SIG
Rate of appressoria formation	-0.006	15	NOT SIG
Rate of penetration	-0.238	14	NOT SIG
PR1	0.233	16	NOT SIG
PR2 + PR4	0.053	16	NOT SIG
PR4	0.181	16	NOT SIG
PR2	-0.261	16	NOT SIG
Ratio PR1:PR2 + PR4	0.051	16	NOT SIG
Ratio PR2:PR4	-0.116	16	NOT SIG

DISCUSSION

A high rate of germination correlated with virulence for the desert locust *Schistocerca gregaria*, among 19 *Metarhizium anisopliae* and *flavoviride* isolates. In other insect hosts the rate of germination has been seen to be a pathogenicity factor of other *M. anisopliae* isolates. Hassan *et al.* (1989) showed that conidia of isolate Me1 soaked in distilled water had an increased rate of germination and an increased pathogenicity towards *Manduca sexta* larvae. In mosquito larvae, *M. anisopliae* isolates that germinated rapidly were more pathogenic than isolates that germinated slowly (Al-Aidroos and Roberts, 1978) both *in vitro* and *in vivo*. Samuels *et al.* (1989) also found that rapid germination *in vitro* (as measured by time to 50% germination) was correlated with high pathogenicity of isolates of *M. anisopliae* for *Nilaparvata lugens*.

The rate of germination has also been shown to be positively correlated with virulence for other entomopathogenic fungi. Jackson *et al.* (1985) found traits significantly contributing to, or associated with, the expression of virulence of eighteen isolates of *Verticillium lecanii* to the aphid *Macrosiphoniella sanborni*. These traits included a fast rate of germination (as measured by time to 50% germination). They also found that the traits acted additively, the absence of one factor not necessarily being detrimental to the isolate's virulence.

Observations of *Beauveria bassiana* infection of *Heliothis zea* showed that highly pathogenic isolates germinated rapidly, but low pathogenic isolates had large clusters of non-germinated spores (Pekrul and Grula, 1979). Hall (1984) measured the epizootic potential of *V. lecanii* infecting the aphid *M. sanborni* as the proportion of the progeny acquiring infection from treated adults. He found that those isolates with a strong epizootic potential also all had fast germination rates, and generally the isolates with low germination rates had reduced epizootic potential.

Germination is seen to be a very important stage in the process of fungal infection. Germination must occur successfully before the penetration process can begin. It is not surprising that the rate of germination is seen to be a determining

factor in virulence as the longer an ungerminated spore is on the surface of a host the greater its chances of being dislodged. Spores that germinate rapidly will become firmly attached sooner, and will be less likely to be removed by preening or dislodged by foliage or other insects brushing the insect host. The spores are also susceptible to desiccation, being sloughed off during ecdysis, and antibiosis from saprophytic microbes (Charnley, 1984). Another advantage of rapid germination is that the spores will be exposed to harmful UV radiation from the sunlight for shorter periods.

A high rate of germination will also produce a more synchronous germination, which may lead to a more synchronous penetration of the insect host. This 'mass action' effect may cause greater stress to the insect's defences and so lead to increased virulence of fast germinating isolates in this way (Dillon and Charnley, 1985). In the present work those isolates that do not reach 50% germination by 12 h but started germinating at 8 h at the latest are obviously much less synchronous. The potential importance of more synchronous germination of spores was noted by Smith and Grula (1981) who attempted to, but did not succeed, in 'shocking' *B. bassiana* conidia into synchronised germination by heat and enzyme treatment.

Although germination is a prerequisite for further prepenetration stages and penetration itself, it is not automatic that these later stages will occur. The environment of the germinating spore and the nutrients available must be correct. Boucias and Latgé (1988) found that using various cuticle extracts from host and non-host insects they could induce germination of *Conidiobolus obscurus* and *Nomuraea rileyi*, but only extracts from host cuticle supported continued germ tube growth. They suggested that the chemical triggers for germination were present, but that the nutritional requirements for continued germ tube development were absent. St. Leger *et al.* (1992) showed that the nutritional requirements for germination and appressoria formation were frequently host related, irrespective of genetic and geographical 'unrelatedness'.

Boucias and Latgé (1988) also noted that *N. rileyi* produced microconidia in situations where, they suggested, the chemical triggers for germination were present,

but that the nutritional requirements for continued germ tube development were absent. They were able to induce the microconidial cycle in *N. rileyi* by placing conidia with short germ tubes in a nutrient limiting media. This is similar to what was seen with *M. flavoviride* isolates Arsef 2023 and later 324673 and 330189. Microcycle sporulation is seen to occur in a number of fungi, for example *Aspergillus niger*, *Penicillium urticae* and *Neurospora crassa*, and can take on a range of forms from conidia producing apparatus that is considerably modified or reduced, as in *P. urticae*, to conidiophores that are little different from those usually seen, as in *A. niger* (Anderson, 1978). The conidia produced here by the three *M. flavoviride* isolates appeared very similar to those produced on solid media, although they were not in strings as those on solid media are but were all individually attached to the hyphal tips where they formed. The Entomophthorales are also seen to produce conidia in situations where inappropriate signals for differentiation are received, but these conidia are termed secondary conidia as they arise directly from the primary conidia with no hyphal growth (Samson *et al.*, 1988). This is not what was seen with the three *M. flavoviride* isolates, where definite hyphal growth occurred.

It is possible that the cuticle of the locust wing contained the correct triggers for germination to occur with the three *M. flavoviride* isolates, but was sufficiently different form locust body cuticle to have incorrect cuticle components available for continued germ tube growth, although 324673 and 330189 did eventually go on to produce appressoria and penetrate the wing, possibly following germination of the microconidia (although this was not investigated). However, microcycle conidiation of 330189 has not been observed on wings *in vivo*. Seyoum (1994) and Bath (personal communication) found normal germination followed by appressorial formation on wings still attached to the locust. Thus *post mortem* changes in wing cuticle chemistry and/or the effects of propylene oxide may make the wing cuticle sub-optimal for certain isolates of *Metarhizium* resulting in microcycle conidiation.

The least pathogenic isolates tended to have long, unbranched germ tubes and more than one germ tube per spore before any appressoria formed, and had

subterminal appressoria. The highly pathogenic and intermediate isolates produced, at least initially, only terminal appressoria on relatively short germ tubes (<10 times spore length). Other authors have discussed germ tube length with regard to pathogenicity. Observations of *B. bassiana* growth on *H. zea* showed that highly pathogenic isolates produced short germ tubes and penetrated rapidly, but low pathogenicity isolates had errant hyphal growth with significant elongation of hyphae before penetration (Pekrul and Grula, 1979). McCauley *et al.* (1968) found great variation in the length of germ tubes of *M. anisopliae* on four species of elaterid larvae and suggested chemo-orientation to specific sites of soft cuticle. Schabel (1978) observed *M. anisopliae* germinating on *Hylobius pales* and found many long germ tubes formed on both soft and hard cuticle and suggested that rather than the fungus looking for 'soft' spots in the cuticle the hyphae instead reach other hyphae, appressoria or infection cushions and fuse with them, increasing the invasive properties of the fungus.

The surface topography is also known to affect the length of *M. anisopliae* germ tubes, according to St. Leger *et al.* (1991b) who showed that on the convoluted surface of less than 1 day old fifth instar *M. sexta* larvae the germ tubes were long, but on the relatively smooth surface of 5 day old fifth instar larvae appressoria formed after the production of only short germ tubes. Butt *et al.* (1992) did not find that surface topography influenced germ tube length and thus appressoria production. *M. anisopliae* germlings on the cuticles of the mustard beetle (*Phaedon cochleariae*) and the cabbage stem flea beetle (*Psylliodes chrysocephala*) were seen to have produced appressoria on germ tubes of varying length in close proximity. The surface of the wing sections used in this study would be expected to be fairly consistent, as they came from locusts of the same age, therefore the differences in germ tube length seen here cannot be attributed to differences in surface topography. The germ tubes certainly did not appear to be growing toward any discernible surface feature.

Butt *et al.* (1992) also found that the appressoria that formed on the cuticles of both beetle species had a range of different morphologies, and that various features

such as sensillae or setae did not appear to induce appressorium formation. They found no obvious relationship between the site of attachment and appressorium induction. The appressoria that formed on the locust wings in this study did not appear to differ noticeably between isolates, and again no surface features could be associated with the initiation of appressoria formation. St. Leger *et al.* (1987b) also found that appressorium formation by *M. anisopliae* on the wings of the blowfly *Calliphora vomitoria* was not related to any discernible surface feature.

No correlation was found between virulence for the desert locust and the rate or time of initial appressoria formation on locust wings, and there was also no correlation between virulence and the rate of penetration. This is the first time that an attempt has been made to correlate the rate of penetration with virulence for any insect/fungus interaction. Butt *et al.* (1992), observing *M. anisopliae* germlings on the cuticles of two beetle species up to 96 hours post inoculation, stated that all the isolates in their study were capable of producing appressoria and thus specificity was determined at a stage after appressorium formation. This would not appear to be the case here as the rate of germination is seen to correlate with virulence. Also differentiation of the germ tubes into appressoria is only likely to occur if the surface conditions are suitable (St. Leger *et al.*, 1992) therefore implying specificity. As with germination, appressoria formation is a prerequisite for penetration and, although no correlation between the timing or rate of appressoria formation and virulence was found, without appressoria formation pathogenesis could not occur. The same is true of penetration. They are both essential steps in the development of mycosis.

It is interesting to note that although there was a wide range of times of initial appressoria formation for the 18 isolates studied, by the stage of penetration there was a much narrower range of times of initial penetration. This may explain why there was no correlation between the time of initial appressoria formation and virulence. The isolates all appear to penetrate the cuticle at a similar time post inoculation, irrespective of the time of initial appressoria formation. This possibly implies that the isolates vary in their ease of penetration. The isolates that take longer to penetrate

after forming appressoria are those that have more difficulty in penetration the cuticle. As can be seen from the enzyme assays the amounts of proteases produced by each isolate vary greatly, and it is possible that some isolates take longer to penetrate after appressoria formation than others due to a lower level of production of the necessary cuticle-degrading enzymes.

The rate of penetration of the cuticle should *a priori* be an important factor in the virulence of an isolate as the more penetrant hyphae, and the more quickly they arrive in the haemocoel, the greater the job of the insect's immune system to overcome them. One isolate may be more effective than another if it penetrates the host more synchronously.

Only individual spores and clumps of up to five spores and their associated penetration points were counted, but penetration also occurred from large clumps of spores. This could be an important factor in oil formulations as the oil and therefore the spores will tend to clump in cuticular folds and leg joints, and from these points a 'mass penetration' could occur. Indeed Prior *et al.* (1988) stated that one of the advantages of oil formulations was that the cutinophilic properties of the oil allowed a much larger number of conidia to reach the more susceptible intersegmental membranes. However, in practice this clumping and mass penetration turns out not to be an advantage. Seyoum (1994) found that by spraying locusts with an oil formulation of *M. flavoviride* isolate 330189, from a ULV track sprayer, a dose of only 200 conidia/locust was required to achieve greater than 80% kill by day 8, whereas with a point inoculation 75,000 conidia/locust were required to achieve the same level of kill. This shows that the fungus is more effective, in this case requiring 300 fold less spores, when applied to the whole body of the locust than in a single, clumped application.

Locust wings are known to be constructed of a framework of fine veins which are hollow sclerotised cuticular tubes through which blood, tracheae and nerves connect with the body cavity. Between these veins are membranes composed of two thin cuticular layers that have a space between them in which blood can circulate

(Uvarov, 1966). Schabel (1978) studying *M. anisopliae* infecting *H. pales*, the pales weevil, found that penetrant hyphae from appressoria on the metawings grew and spread within the wings. In this study all the *M. anisopliae* isolates were seen to be able to penetrate the cuticle of the wing and the resulting penetrant hyphae then grew out within the wing space. Kennedy *et al.* (1948) sprayed locusts in flight with 0.45 mm to 0.2 mm drops of insecticide. The wings collected about half of the total drops collected by the whole locust. More recently Seyoum (1994) has found that 51% of an oil formulation of *M. flavoviride* conidia, sprayed by ULV track sprayer, landed on the wings of flying locusts. Thus the wings may be an important route for infection from a mycoinsecticide in the field.

Penetration of the cuticle by *M. anisopliae* is known to occur due to both mechanical pressure of the penetration peg and also the action of cuticle degrading enzymes (Charnley, 1984; Goettel *et al.*, 1989). Goettel *et al.*, (1989) showed that *M. anisopliae* penetrating *M. sexta* cuticle utilised only cuticle degrading enzymes to breach the epicuticle but required both the action of enzymes and the physical separation of lamellae by penetrant hyphae to get through the procuticle. St. Leger *et al.* (1987b) detected the proteolytic enzymes PR1 and PR2 from *M. anisopliae* isolate Me1 on blowfly wings 16 h after inoculation, which was coincident with appressoria formation. In this study the enzymes PR1, PR2 and also PR4 were detected for all *M. anisopliae* isolates and some of these enzymes were detected for two of the three *M. flavoviride* isolates, at the time of initial penetration for each individual isolate. No correlation was found between the levels or ratios of these enzymes for each isolate and virulence for the desert locust.

Attempts have been made by other authors to correlate virulence of *M. anisopliae* isolates, and other entomopathogenic fungi, with the production of cuticle degrading enzymes, but all previous studies have involved assaying enzyme production on artificial media. Samuels *et al.* (1989) found no direct correlation between *in vitro* enzyme production and pathogenicity of *M. anisopliae* towards *N. lugens*, but chitinase, lipase and protease activity together with a lack of amylase

activity were features of highly pathogenic isolates. No correlation was found between proteolytic activity and virulence of *M. anisopliae* for mosquitoes (Al-Aidroos and Seifert, 1980) or *Rhodnius prolixus* (Silva and Messias, 1986) although both groups found a relationship between amylase production and virulence. Samsinakova and Misikova (1973) found that *M. anisopliae* as well as *B. bassiana* and *Aspergillus parasiticus* isolates with the highest infectivity towards *G. mellonella* larvae produced the highest amount of chitinase. Low virulence strains of *B. bassiana* for *G. mellonella* had low lipolytic and proteolytic activity while high enzyme activity was seen in high virulent strains (Pavlyushin, 1978). Gupta *et al.* (1994), also observing *in vitro* enzyme production of *B. bassiana* with regard to *G. mellonella*, found that the onset of mortality was related to high levels of chymoelastase, chymotrypsin and endochitinase, and the rate of mortality was related to high esterase and N-acetylglucosaminidase levels. Bidochka and Khachatourians (1990) determined that a protease produced by *B. bassiana* was a virulence factor, as a mutant with reduced protease production extended the time course of pathogenesis of the fungus on the grasshopper *Melanoplus sanguinipes*.

El-Sayed *et al.* (1989) found chitinolytic activity in both virulent and avirulent isolates of *N. rileyi* towards *Trichoplusia ni*, but virulent isolates consistently contained significantly higher levels of chitinase. Jackson *et al.* (1985) found a number of traits significantly contributing to or frequently associated with the expression of virulence of *V. lecanii* to *M. sanborni*, and these included an absence of extracellular amylase activity and a relatively high extracellular chitinase production. They concluded that a number of traits act additively towards virulence. Leathers and Gupta (1993) concluded that cuticle-degrading enzymes may not be the limiting factor in virulence of *B. bassiana* for the eastern tent caterpillar, *Malacosoma americanum*, as highly virulent strains were not exceptional in their *in vitro* production of cuticle-degrading enzymes. Champlin *et al.* (1981) also found that of the mutants of *B. bassiana* they were studying all but one produced significant

amounts of extracellular enzyme activities, proteolytic, chitinolytic and lipolytic activity, but there was no positive correlation with the killing of the pecan weevil.

In this study only a correlation was found between the virulence of *M. anisopliae* isolates for the desert locust, *S. gregaria*, and the rate of germination of the isolates used, but not for any of the other parameters measured. However, the cuticle is a major barrier to infection and it seems, *a priori*, to be an important structure affecting the virulence of fungal pathogens. The importance of the ability of a fungus to invade the cuticle was shown by Ferron and Diomandé (1969) who found that only *M. anisopliae* strains isolated from *Orycetes* spp. could, when topically applied, infect *Orycetes monoceros*, isolates from other species being pathogenic only on injection.

Many authors have suggested that virulence is not determined by a single factor such as a particular cuticle-degrading enzyme. Although Paris and Ferron (1979) found a relationship between virulence and lipase production in *B. brogniartii* mutants they also found mutants deficient in other respects were avirulent, and so suggested that pathogenicity was determined by more than one factor. Pekrul and Grula (1979), from studies of *B. bassiana* and *H. zea*, suggested that low entomopathogenicity was not simply a consequence of a lack of the right enzymes required for penetration. Heale *et al.* (1989) suggest that only a limited value can be assigned to any one cuticle-degrading enzyme trait amongst the many others which interact and summate in the expression of pathogenicity. Comparisons between isolates for pathogenicity and production of enzymes may, according to Charnley and St. Leger (1991), only reveal the great variability within a species for numerous factors, many of which may influence but be unrelated to cuticle-degrading enzyme activity. This conclusion is supported by the results of the present study, despite the use of a much more reliable methodology than has been employed with such experiments hitherto.

It would appear that the next step in the search for possible virulence and/or pathogenicity determinants of *Metarhizium* spp. against the desert locust or other

hosts is to use recombinant DNA technology. The diverse genetic background of fungal species makes specific approaches, such as site-directed mutagenesis, the best option for identifying single virulence and/or pathogenicity determinants. Once putative virulence/pathogenicity determinants have been identified the corresponding gene(s) should be cloned. Transformation mediated gene disruption will produce a single lesion mutant for the factor under investigation. Restoration of pathogenicity/virulence to these mutants by transformation with intact cloned gene(s) will provide definitive proof which has not been forthcoming from the type of approach adopted in the present work.

CHAPTER 3. INHIBITION OF *SCHISTOCERCA GREGARIA* MALPIGHIAN TUBULE FUNCTION BY DESTRUXINS *IN VITRO.* *

INTRODUCTION

Destruxins were originally isolated on the basis of their ability to cause immediate paralysis of silkworms (Kodaira, 1961). This effect in lepidopteran muscle is due to calcium dependent depolarisation of muscle cell membranes (Samuels *et al.*, 1988b). However, not all insects are susceptible to destruxin-induced paralysis, and other toxic effects of destruxins may also be important during *Metarhizium* mycosis. Vey and Quiot (1989) observed that the Malpighian tubules of waxmoth larvae, *Galleria mellonella*, were particularly sensitive to the cytotoxic effects of destruxins, showing adverse effects on ultrastructure after injection of destruxins.

Now that *Metarhizium* spp. are being developed as biological control agents for desert locusts (Prior *et al.*, 1992), it is of particular interest to investigate the effects of destruxins on *S. gregaria*. While destruxins are not toxic to *S. gregaria* when tested acutely (Samuels *et al.*, 1988a), Huxham *et al.* (1989) have shown that injections of destruxins depress the cellular immune responses of desert locusts. In this chapter the effects *in vitro* of a number of pure destruxins on the function of the Malpighian tubules of *S. gregaria* are investigated.

* - Adapted from James *et al.* (1993).

MATERIALS AND METHODS

Freshly fed adult female locusts between 4 and 14 days after ecdysis were used for experiments. The rate of production of primary urine by *S. gregaria* Malpighian tubules *in vitro* was measured as originally described by Maddrell and Klunsuwan (1973), and modified by Anstee and Bell (1975). Briefly, the gut and associated Malpighian tubules were removed intact from the locust and maintained in a temperature-controlled bath (28°C), bathed in locust saline solution (see Appendix 1), the whole preparation being under the surface of a liquid paraffin (mineral oil) bath. In each preparation ten Malpighian tubules were gently pulled out of the saline solution into the oil and looped around a peg. An incision made in each of these tubules allowed the escape of primary urine into a spherical droplet in the oil. Not all of the tubule loops secreted fluid; those that did not were ignored in subsequent analysis. Where a droplet was formed, its diameter was measured at intervals using an eyepiece graticule in a dissecting microscope in order to calculate its volume. Measurements were made on a number of droplets at 5 min intervals over a 40 min observation period, following which the saline solution in the bath was replaced. Rates of fluid secretion were determined from linear regressions of the volumes of droplets against time. For each treatment, mean rates of fluid secretion were calculated from a minimum of 15 tubules taken from 2 - 4 locusts.

It was not possible to ensure complete exchange of old medium for new when replacing the saline solution bathing the gut and Malpighian tubules. The efficiency of exchange was determined in a separate experiment with the dye, Trypan blue, to be 80%. Concentrations of chemicals quoted have been corrected for this factor.

Destruxins were dissolved directly in locust saline prior to testing. Synthetic *Locusta migratoria* diuretic peptide (sequence given by Kay *et al.*, 1991) was diluted in locust saline from a stock solution (3.8 μM) in methanol. The final concentration of methanol was 1%. Control saline included methanol but no peptide.

RESULTS

Inhibition of fluid secretion by destruxin A.

S. gregaria Malpighian tubules secreted primary urine *in vitro* at a mean rate of 5.71 ± 0.65 nl/min during an initial 40 min experimental period, in which the rate of secretion of individual tubules remained constant (Fig. 1A). Following a change of the bathing medium and a 20 min acclimation period the rate was slightly but not significantly less (4.53 ± 0.54 nl/min).

When tubules were incubated according to the same protocol first in locust saline solution and subsequently in locust saline containing destruxin A, the rate of secretion by the toxin treated tubules was decreased. An example of a single tubule exposed to 80 μM destruxin A is shown in Fig. 1B. The rate of fluid secretion by tubules exposed to the toxin was reduced, but constant during the 40 min experimental period. Inhibition of fluid secretion was dose-dependent (Fig. 2). The concentration of destruxin A required to give 50% inhibition (IC_{50}) was estimated to be 23 μM after allowing for the approximately 10% decrease in secretion that occurred during the experimental period even when destruxin was absent.

Activity of other destruxins.

Four pure destruxins were tested. All inhibited fluid secretion to a similar extent (Fig. 3). There were no significant differences between the secretory rates of tubules exposed to the four toxins at a concentration of 16 μM (ANOVA, $F = 0.64$, $P = 0.7$, $df = 69$).

Partial reversibility of destruxin inhibition.

To assess the reversibility of the inhibition of fluid secretion by destruxin A, Malpighian tubules were incubated first in locust saline solution (30 min), then exposed to 80 μM destruxin A (30 min), subsequently washed twice in destruxin A-free saline, then incubated for 30 min in saline solution (Fig. 4). A significant

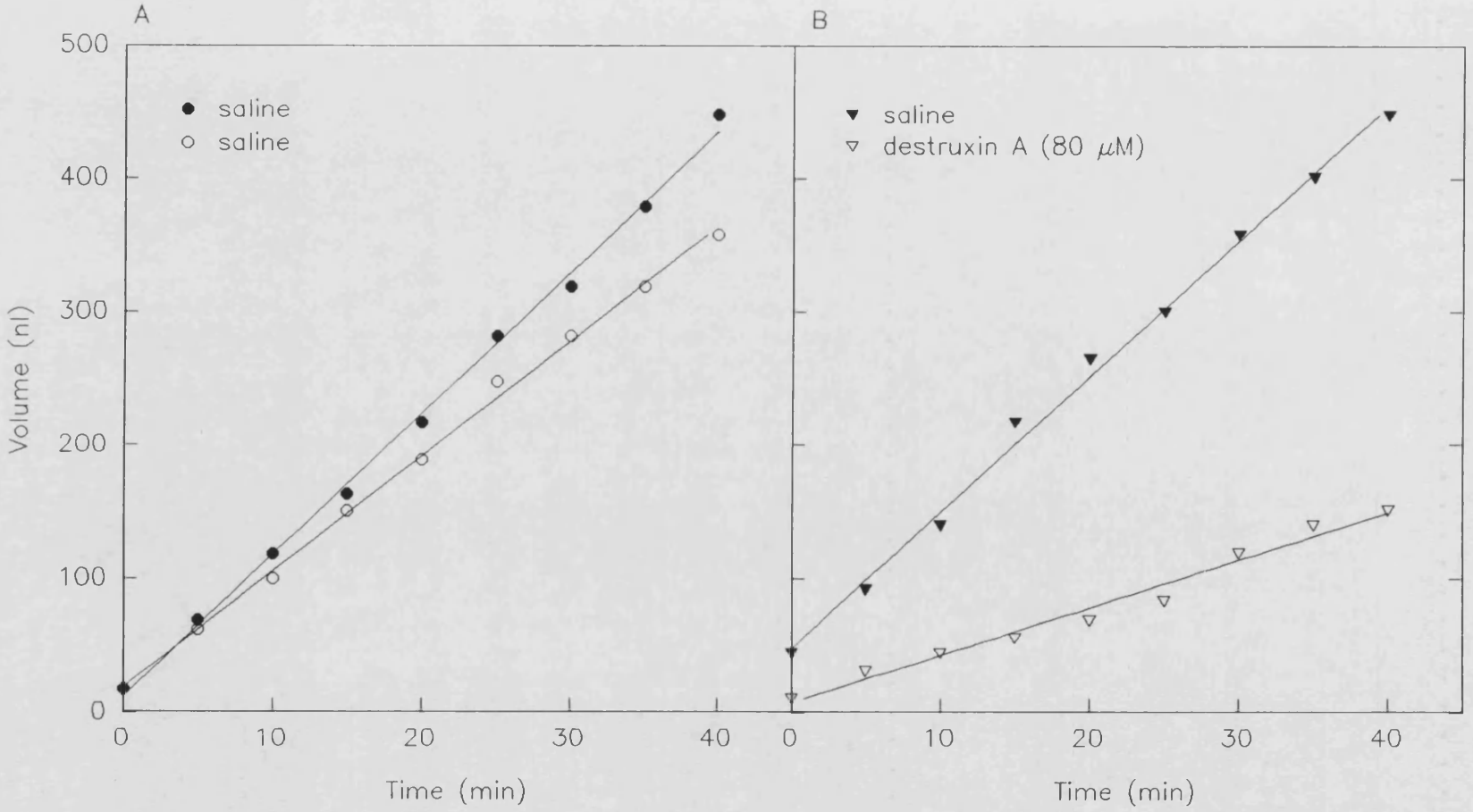


Figure 1.

Fluid secretion by S. gregaria Malpighian tubules is inhibited by destruxin A. Each panel shows the cumulative secretion of fluid by a single Malpighian tubule with time. Solid symbols (● , ▼) show initial control experiments in which the tubule was bathed in locust saline. Open symbols show (A) a control experiment in which the tubule was again bathed in saline (○) ; (B) inhibition by 80 μM destruxin A (▽).

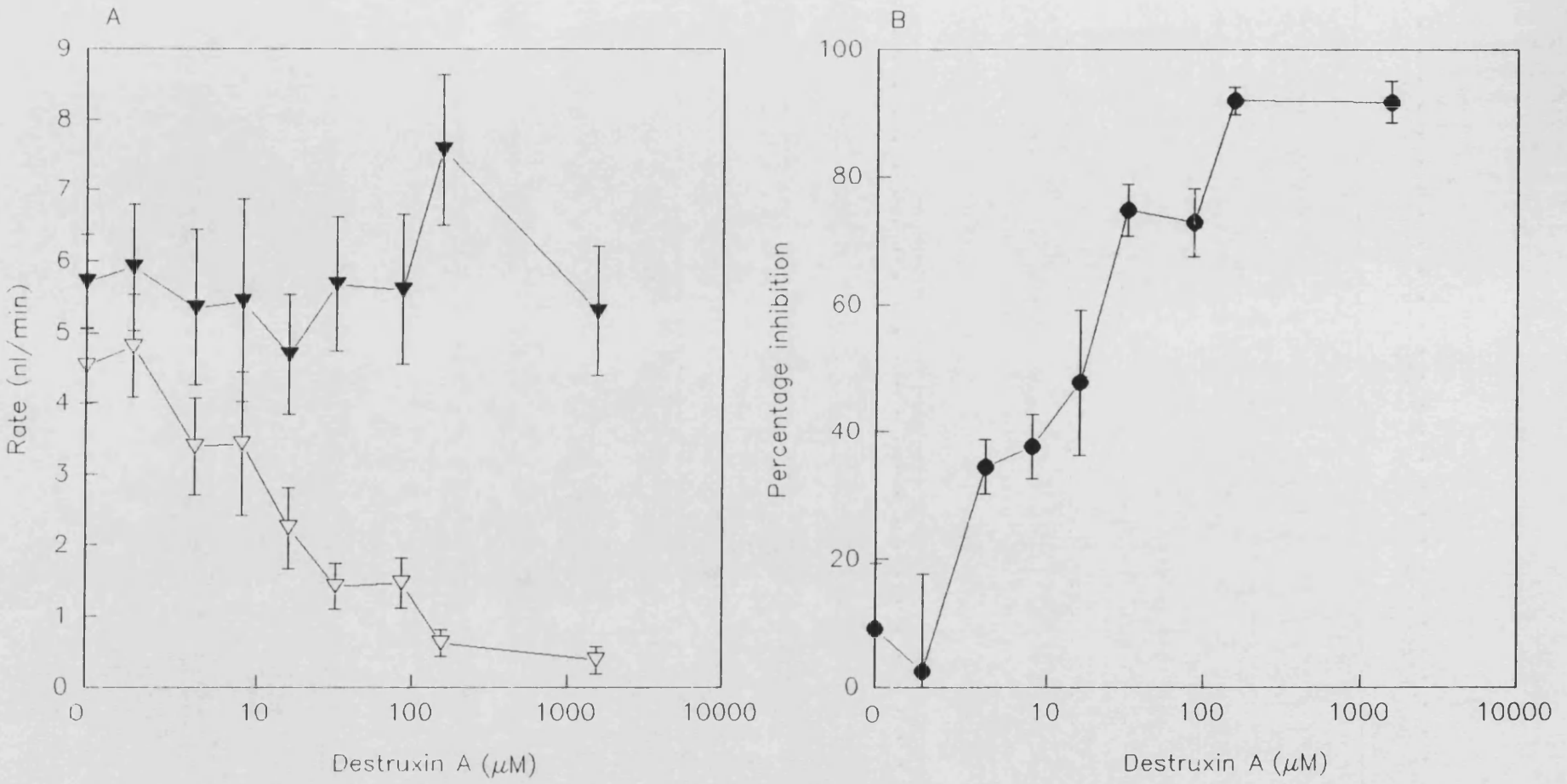


Figure 2.

Dose–response relationship for inhibition of fluid secretion by destruxin A. (A) Rates of fluid secretion before (▼) and after (▽) application of destruxin A. (B) Percentage inhibition calculated from the data in (A). Means ± S.E. 16–22 tubules were used for each point. Each tubule was used to determine a control (saline) rate of secretion, and was then exposed to only a single concentration of destruxin A.

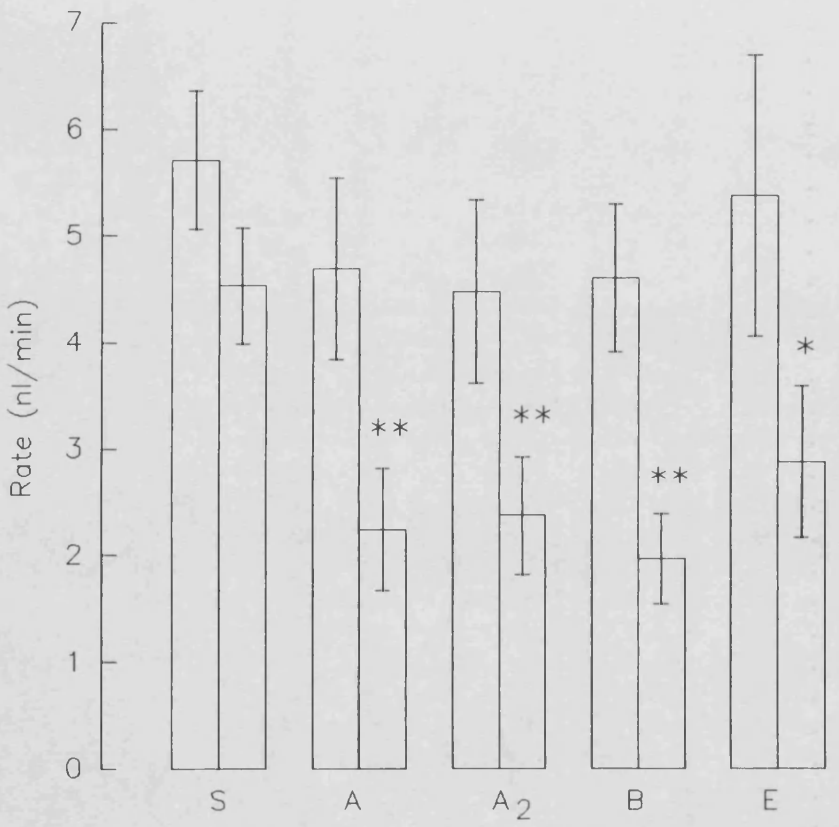


Figure 3.
Inhibition of fluid secretion by different destruxins. For each pair of bars, the left–hand bar shows the initial (control) rate of fluid secretion in the presence of locust saline. The right–hand bar of each pair shows the (test) rate of secretion in the presence of (S) replacement locust saline; (A) destruxin A (16 μM); (A_2) destruxin A_2 (16 μM); (B) destruxin B (16 μM); (E) destruxin E (16 μM). Means ± S.E. 16–19 tubules in each case. For each treatment the initial (control) rate was compared with the subsequent test using a paired t–test. * = $p<0.05$; ** = $p<0.01$.

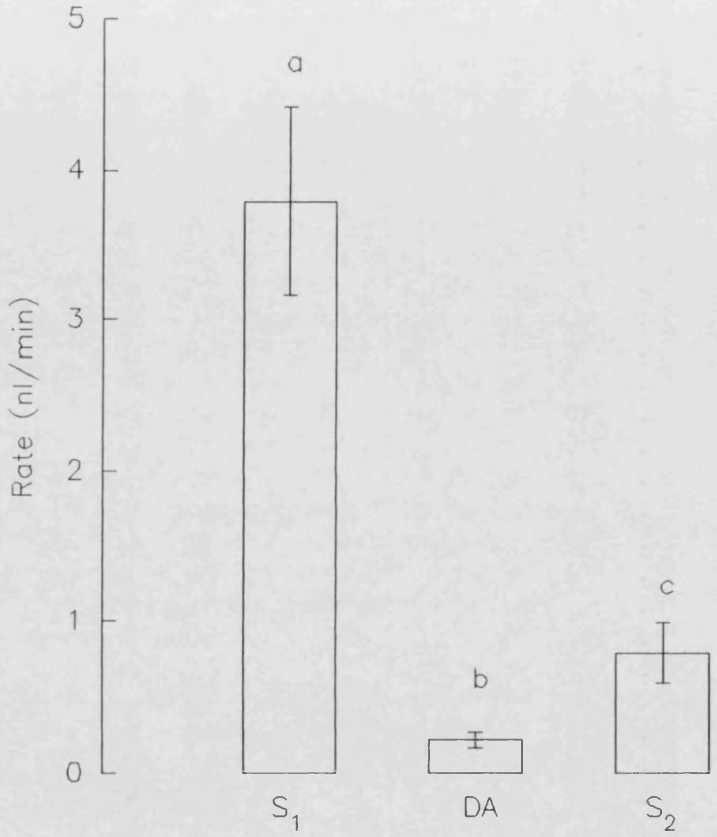


Figure 4.
Partial reversability of inhibition by destruxin A. Fluid secretion was measured initially (S_1) in the presence of locust saline (30 min), and subsequently (DA) in the presence of destruxin A (80 μM) (30 min), at the end of which time the tubules were washed twice with locust saline. Fluid secretion was now measured in the presence of toxin-free locust saline (S_2) (30 min). Mean ± S.E. 21 tubules were used in each case. Rates were compared using a paired t-test. Significant differences ($p<0.01$) are indicated by different letters.

recovery in the rate of fluid secretion was observed when destruxin was removed, although the rate of secretion was significantly less than it had been prior to the toxin treatment. Additional washes did not lead to improved recovery (data not shown).

Response of S. gregaria *Malpighian tubules to* Locusta *diuretic peptide.*

Locusta diuretic peptide significantly increased the rate of fluid secretion *in vitro* by *S. gregaria* Malpighian tubules when added to the bathing medium at a concentration of 30 nM. This enhanced rate of fluid secretion, as well as the resting rate of secretion, were almost completely abolished (93% inhibition) when the tubules were exposed to the same concentration of *Locusta* diuretic peptide in the presence of 80 μM destruxin A (Fig. 5).

Response of S. gregaria *Malpighian tubules to cAMP.*

S. gregaria Malpighian tubules were exposed to 4 mM adenosine 3',5'-cyclic monophosphate (cAMP) in the presence of 0.8 mM 3-isobutyl-1-methylxanthine (IBMX) (an inhibitor of cAMP phosphodiesterase). This treatment resulted in a significantly enhanced rate of fluid secretion. When tubules were incubated first in saline solution containing 80 μM destruxin A, and then in saline containing both destruxin A (80 μM) and the cAMP (4 mM)/IBMX (0.8 mM) mix, the destruxin-treated tubules failed to respond to the cAMP treatment, continuing to secrete at a rate significantly less than the normal resting rate (Fig. 6).

Effects of calcium-free medium and cadmium ions.

S. gregaria Malpighian tubules secreted apparently normally in the absence of external calcium. When the tubules were bathed in a modified locust saline without calcium salts and containing 1 mM ethylene glycol-bis(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), the rate of fluid secretion was similar to the normal basal (unstimulated) rate over two successive 40 min periods (Fig. 7).

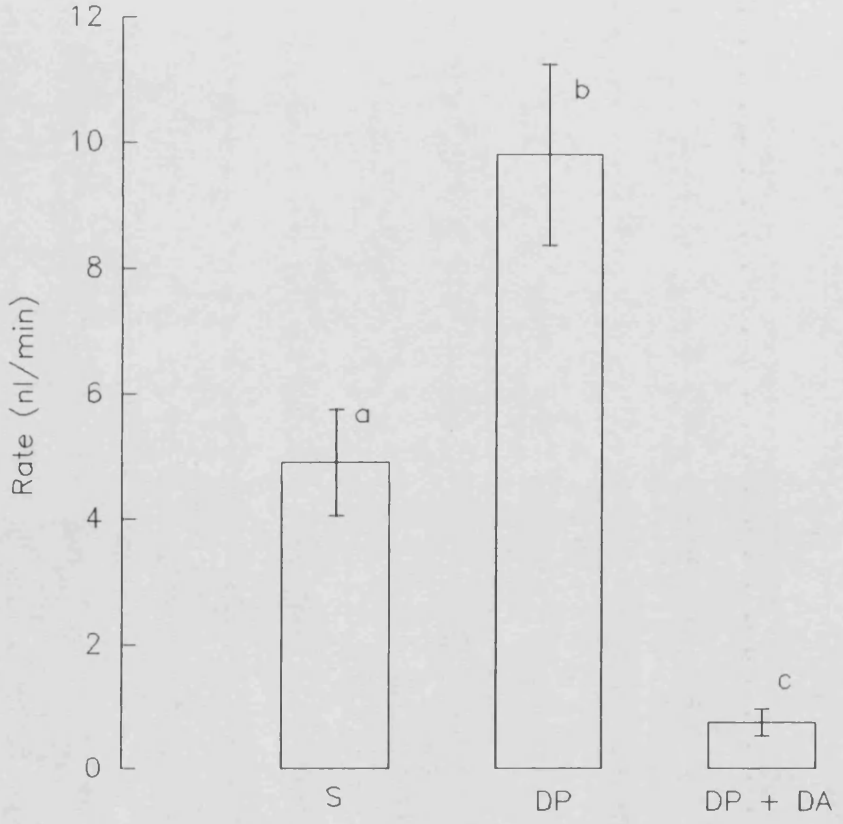


Figure 5.
Destruxin A inhibits fluid secretion stimulated by Locusta migratoria diuretic peptide (Lom DP). For each tubule, fluid secretion was measured during successive 30 min periods in the presence of (S) locust saline, (DP) Lom DP (30 nM), and (DP + DA) Lom DP (30 nM) and destruxin A (80 μM). Mean ± S.E. 18 tubules used in each case. Rates were compared using a paired t−test. Significant differences ($p<0.01$) are indicated by different letters.

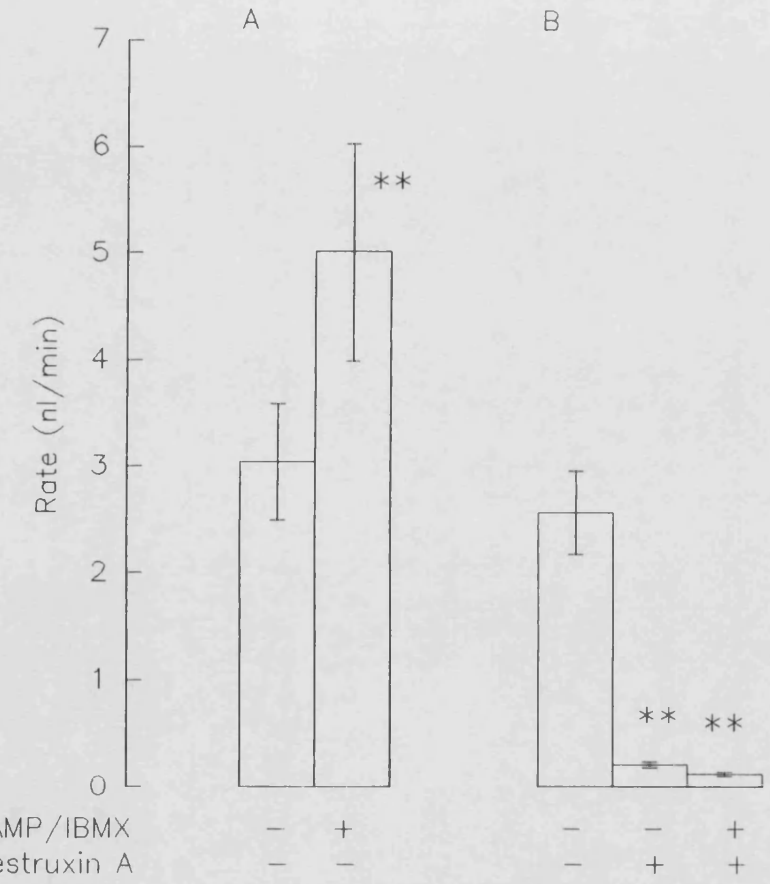


Figure 6.

Destruxin A inhibits fluid secretion stimulated by cAMP. (A) Fluid secretion was stimulated when cAMP (4 mM) was added in the presence of the phosphodiesterase inhibitor IBMX (0.8 mM). (B) Preincubation with destruxin A (80 μM) prevented cAMP stimulation of fluid secretion. Means ± S.E. 22 − 23 tubules in each caes. Rates were compared using a paired t−test. Significant differences from the control (i.e. no cAMP/IBMX, no destruxin A) are indicated as * = $p<0.05$, ** = $p<0.01$.

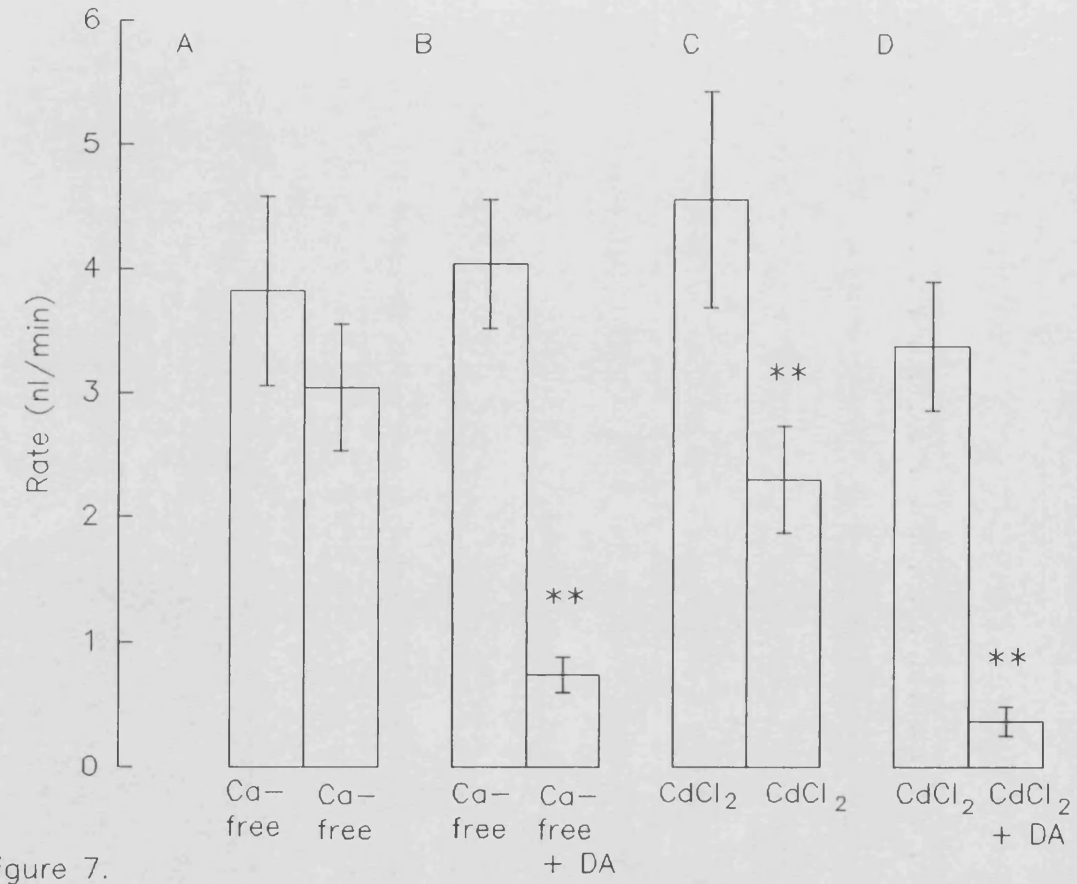


Figure 7.

Calcium, cadmium and the action of destruxin A. (A) Calcium–free locust saline (containing 1mM EGTA) does not significantly inhibit fluid secretion; (B) 80 μM destruxin A (DA) inhibits fluid secretion in the absence of external calcium; (C) cadmium chloride (0.25 mM) in calcium–free locust saline (without EGTA) significantly inhibits the resting rate of fluid secretion; (D) in the presence of 0.25 mM cadmium chloride, destruxin A (80 μM) causes further significant inhibition of fluid secretion. [N.B. In experiment (C) and (D) the locust saline was buffered with HEPES instead of sodium phosphate]. Means ± S.E. 16 – 24 tubules in each case. Experimental treatments were compared with the corresponding control using a paired t–test. ** = $p<0.001$.

The absence of calcium ions did not affect the ability of destruxin A to inhibit fluid secretion, which was reduced by 80% when 80 μM destruxin A was present in the calcium-free medium.

Cadmium ions, which block calcium channels, have previously been reported to block destruxin action (Samuels *et al.*, 1988b). The effect of cadmium on destruxin A inhibition of *S. gregaria* Malpighian tubules was tested by including 0.25 mM $CdCl_2$ in a slightly modified saline solution (it was necessary to replace the phosphate buffer of the Maddrell and Klunsuwan *S. gregaria* saline by 5 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) to avoid precipitation of cadmium). Cadmium ions were deleterious to Malpighian tubule function, so that although the rate of fluid secretion was normal during an initial 40 min exposure, the rate of secretion declined significantly during the second 40 min period of cadmium treatment. However, cadmium did not prevent destruxin inhibition of fluid secretion, which occurred normally (Fig. 7).

Effects of SITS.

Cerenius *et al.* (1990) reported that the stimulatory effects of destruxin A on the degranulation of crayfish haemocytes were abolished by the anion channel blocker, 4-acetamido-4'-isothio-cyanatostilbene-2,2'-disulphonic acid (SITS). At 8 mM, a similar concentration to that used by Cerenius *et al.* (1990), this chemical proved to be an inhibitor of resting fluid secretion in *S. gregaria* Malpighian tubules (see Fig. 8A). However even at this concentration, SITS failed to prevent the further inhibition of fluid secretion by 80 μM destruxin A (Fig. 8B).

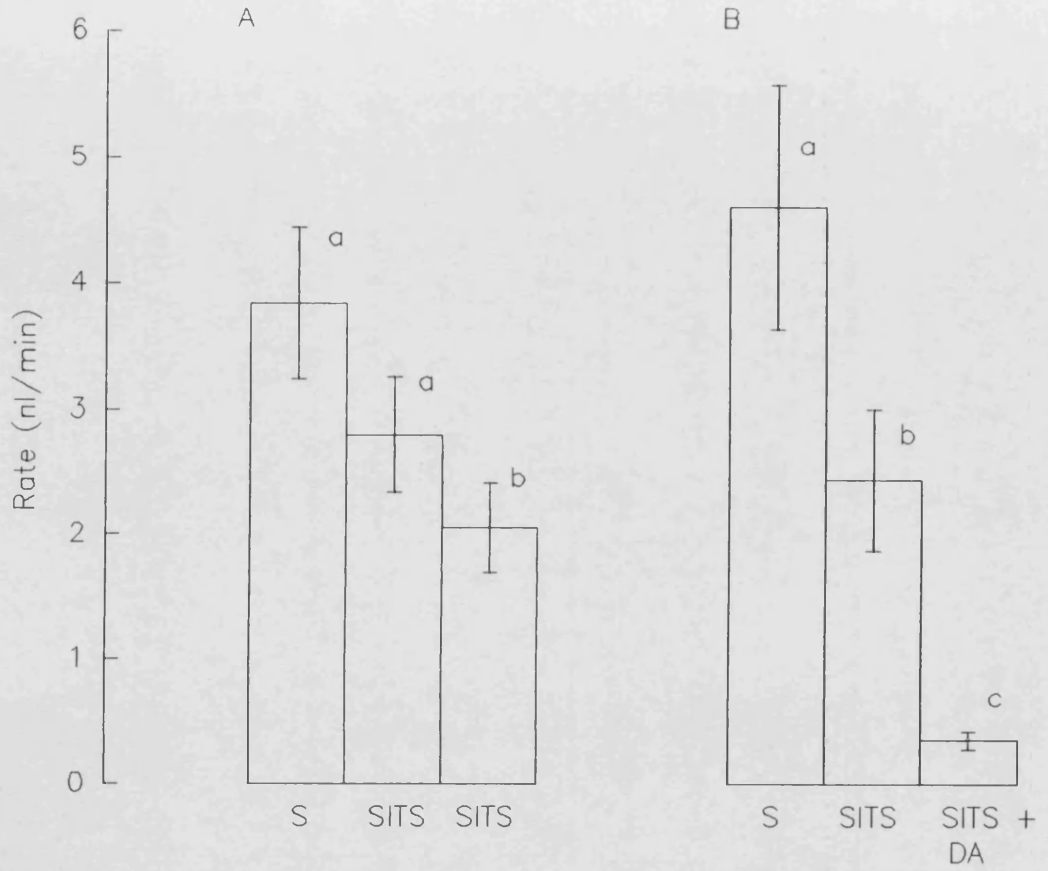


Figure 8.
Effect of SITS on fluid secretion and on destruxin A action. (A) Malpighian tubules were incubated for successive 30 min periods with (S) locust saline, and subsequently with 2 changes of SITS (8 mM); (B) tubules were incubated successively with saline (S), SITS (8 mM), and SITS (8 mM) plus destruxin A (80 μM) (DA). Means ± S.E. (A) 23 tubules; (B) 15 tubules. Means were compared using a paired t–test. Significant differences ($p<0.01$) within each experiment are indicated by different letters.

DISCUSSION

Inhibition of fluid secretion by destruxins.

Pure destruxins A, A_2, B and E are all seen to inhibit the resting rate of fluid secretion by desert locust Malpighian tubules. For destruxin A the IC_{50} was approximately 23 μM. While complete dose-response curves were not determined for the other destruxins, their inhibitory effects at the single dose of 16 μM were not distinguishable from that of destruxin A.

Inhibition of secretion by destruxin A appears to be at least partially reversible, since there was a significant recovery in the rate of fluid secretion when the toxin was removed after 30 min exposure. However, recovery was not complete. It is possible that exposure of Malpighian tubule cells to destruxins may cause permanent damage. Vey and Quiot (1989) observed cytological changes in Malpighian tubule cells of *G. mellonella* after destruxin treatment *in vivo,* and similar changes have also been observed in the Malpighian tubules of the blowfly *C. vomitoria* after destruxin injections (S.R. Watson and A.K. Charnley, unpublished). Destruxins are known to have cytotoxic effects on cultured insect cells (Vey and Quiot, 1989; Kershaw, 1993). It is not clear whether these cytotoxic effects of destruxins are directly related to the inhibition of fluid secretion described here.

Stimulation of fluid secretion and its inhibition by destruxins.

Fluid secretion by desert locust Malpighian tubules is probably regulated by a diuretic hormone present in the corpora cardiaca (Mordue, 1969). The diuretic hormone of *S. gregaria* has not been chemically characterised, but that of the migratory locust, *L. migratoria* has been isolated and sequenced (Kay *et al.*, 1991; Lehmberg *et al.*, 1991), and found to be a 46-residue peptide related in sequence to the corticotrophin releasing factor/sauvagine/urotensin I family of peptides. The peptide stimulates fluid secretion in isolated *L. migratoria* Malpighian tubules by a factor of 2-3 times (Lehmberg *et al.*, 1991).

Here the *Locusta* diuretic peptide (Lom DP) was also seen to stimulate fluid secretion by Malpighian tubules of *S. gregaria.* When exposed to a peptide concentration of 30 nM, the rate of secretion was increased to 2.2 times the basal rate. This result suggests that the native diuretic peptide in *S. gregaria* must be very similar to that of *L. migratoria.* Destruxin A inhibited Lom DP-stimulated fluid secretion. 80 μM toxin caused a 93% reduction in secretory rate as compared to the stimulated control.

Mechanism of action of destruxins.

Lom DP probably stimulates fluid secretion by increasing the production of the intracellular second messenger cAMP in Malpighian tubule cells (Morgan and Mordue, 1985). Synthetic Lom DP is known to increase cAMP levels in *L. migratoria* Malpighian tubules (Kay *et al.*, 1991; Lehmberg *et al.*, 1991). Here we show that exogenous cAMP, when administered together with the cAMP phosphodiesterase inhibitor IBMX, causes an increased rate of fluid secretion in *S. gregaria* tubules, just as has been shown in *L. migratoria* (Morgan and Mordue, 1981; 1985).

The inhibition of fluid secretion by destruxins might thus be due to the inhibition by the toxin of cAMP synthesis. However, this seems most unlikely, since destruxin A inhibited cAMP/IBMX-stimulated fluid secretion just as effectively as it inhibited hormone-stimulated fluid secretion. This suggests that the toxin acts at a level beyond that of cAMP control.

Ion channels and destruxin action.

Previous work has suggested that at least some actions of destruxins are calcium-dependent. Depolarisation of *M. sexta* larval body wall muscle was prevented when both calcium and magnesium were removed from the external medium, and was abolished by the calcium channel blockers cadmium chloride and nifedipine (Samuels *et al.*, 1988b). The antagonistic effect of nifedipine on destruxin B action in lepidopteran muscle was confirmed by Bradfisch and Harmer (1990), who

also found that omega-conotoxin GVIA prevented destruxin-induced depolarisation. These findings suggest that voltage-gated calcium channels may be involved in the response to destruxins. Destruxin-induced degranulation of crayfish (*Pacifastacus leniuseulus*) haemocytes is also calcium dependent, being abolished in calcium-free conditions and by cadmium ions (Cerenius *et al.*, 1990).

The inhibition of fluid secretion by destruxins was not, in the present case, calcium-dependent. Calcium-free conditions did not prevent fluid secretion continuing at normal basal rates, nor did they prevent inhibition by destruxin A. Cadmium chloride (used at a concentration adequate to prevent destruxin-induced depolarisation in *M. sexta* muscle) itself caused some inhibition of fluid secretion. However, the use of appropriate controls clearly showed that cadmium ions did not prevent the destruxin inhibition of fluid secretion by *S. gregaria* Malpighian tubules, which occurred normally.

The degranulating effects of destruxins on crayfish haemocytes are also inhibited by the anion channel blocker, SITS (Cerenius *et al.*, 1990). We found that SITS alone caused a progressive reduction in the rate of fluid secretion by *S. gregaria* Malpighian tubules. Again, however, this did not prevent the normal inhibition of fluid secretion by destruxin A.

These two results suggest that neither calcium channels nor anion channels participate directly in the toxic effects of destruxins on Malpighian tubules, at least as far as fluid secretion is concerned. It also suggests that either the mechanism of action of destruxins in *S. gregaria* Malpighian tubules is different to that in caterpillar muscles and crayfish haemocytes, or that in the latter cases these ion channels are only indirectly involved in the expression of the symptoms of destruxin-induced toxicity. A similar conclusion was reached for the inhibition by destruxins of ecdysteroid secretion in *M. sexta* prothoracic glands (Sloman and Reynolds, 1993), where it was suggested that the toxins act at a level beyond the control of either calcium or cAMP.

CHAPTER 4. INHIBITION OF *SCHISTOCERCA GREGARIA* MALPIGHIAN TUBULE FUNCTION BY DESTRUXIN A *IN VIVO.*

INTRODUCTION

Destruxins have been shown to inhibit *S. gregaria* Malpighian tubule fluid secretion *in vitro* (see chapter 3; James *et al.*, 1993). Earlier studies using adult blowfly (*C. vomitoria*) showed that destruxins were able to inhibit Malpighian tubule fluid secretion *in vivo*, indicated by the inhibition of the removal of an injected dye, amaranth, from the insect's haemolymph (S.R. Watkins, J.S. Cook and A.K. Charnley, unpublished). In this section a similar method is employed to investigate the effects of injected destruxin A on *S. gregaria* Malpighian tubule function *in vivo*.

M. anisopliae isolate Me1 is known to produce destruxins during mycosis of *M. sexta* larvae (Samuels *et al.*, 1988a). The role of destruxins in *S. gregaria* pathogenicity is unclear, and the effect of *M. anisopliae* isolate Me1, a known destruxin producer, on the function of *S. gregaria* Malpighian tubules *in vivo* is investigated.

MATERIALS AND METHODS

Freshly fed locusts.

Freshly fed adult male *S. gregaria* (two weeks after ecdysis) were injected with 25 µl of a 2% (w/v) amaranth solution in locust saline, containing 25 µg of destruxin A. Control locusts were injected with 25 µl of a 2% (w/v) amaranth solution in locust saline. The destruxin A was initially dissolved in ethanol and diluted to give 0.5% ethanol in the final injected solution. Corresponding controls also contained 0.5% ethanol in the final injected solution. Six locusts were used in each case. The solution was injected into the third dorsal abdominal sclerite, using a Hamilton syringe. At 5 min intervals over the first 30 min after injection 5 µl haemolymph samples were taken, by piercing the base of the hind leg on the opposite side to injection. Each sample was diluted in 1 ml locust saline and read on a spectrophotometer at 520 nm. The experiment was carried out at 28°C ± 1°C.

Infected locusts.

Each of twelve adult male *S. gregaria* (two weeks after ecdysis) were inoculated under the pronotum with 75,000 spores of *M. anisopliae* isolate Me1 in cotton seed oil. Twelve control locusts were inoculated in the same manner with oil alone. The locusts were then placed in individual containers and kept at 28°C, 30% RH with a 16 h light : 8 h dark cycle for 4 days, with no access to food or water. On day 4 of infection (day 0 being the day of inoculation) 6 control and 6 infected locusts were injected with 25 µl of a 2% (w/v) amaranth solution in locust saline. The solution was injected into the third dorsal abdominal sclerite, using a Hamilton syringe. Haemolymph samples were taken over the first 30 min after injection by piercing the base of the hind leg on the opposite side to injection. Samples were diluted in 1 ml of locust saline and read on a spectrophotometer at 520 nm. The experiment was carried out at 28°C ± 1°C.

RESULTS

Freshly fed locusts.

Injected amaranth is gradually removed from the haemolymph of control and destruxin treated locusts (Fig. 1). This shows the removal to be quite well described by a simple logarithmic relationship, for the first 20 min. After 20 min the rate of removal declined and the mathematical description of the rate of decline in dye concentration was more complex (data not shown). It will be noticed from Fig. 1 that the regression lines for control and destruxin-treated insects project back to different values at zero time. This is considered further in the discussion.

Amaranth concentrations in the haemolymph of individual control and destruxin treated locusts were plotted against time (Figs. 2A and B). The individual rates of removal of amaranth from the haemolymph were calculated from the slopes of the regression lines drawn through each set of data. During the first 20 min after injection, the control locusts had significantly higher rates of removal of amaranth than the destruxin treated locusts, control mean rate = 0.0143 μg/ml/min ± 0.0010 and destruxin treated locusts mean rate = 0.0090 μg/ml/min ± 0.0013 (T-test, T = -3.24, p = 0.01). Thus destruxin A inhibited the initial removal of amaranth from the haemolymph of locusts.

Infected locusts.

The isolate Me1 was used to infect the locusts since it is known to produce destruxins in both liquid culture and mycosed insects (Samuels *et al.*, 1988a). Bioassay results (Appendix V) show that under these conditions the majority of Me1 infected locusts die on day 5, therefore day 4 was taken to be the day on which high levels of destruxins could be present in the blood, whilst leaving sufficient locusts alive to conduct the experiment.

Insufficient haemolymph could be obtained from the Me1 infected locusts to calculate the rates of removal of amaranth from the haemolymph. However, observations during the attempt to collect haemolymph samples indicated that, in the

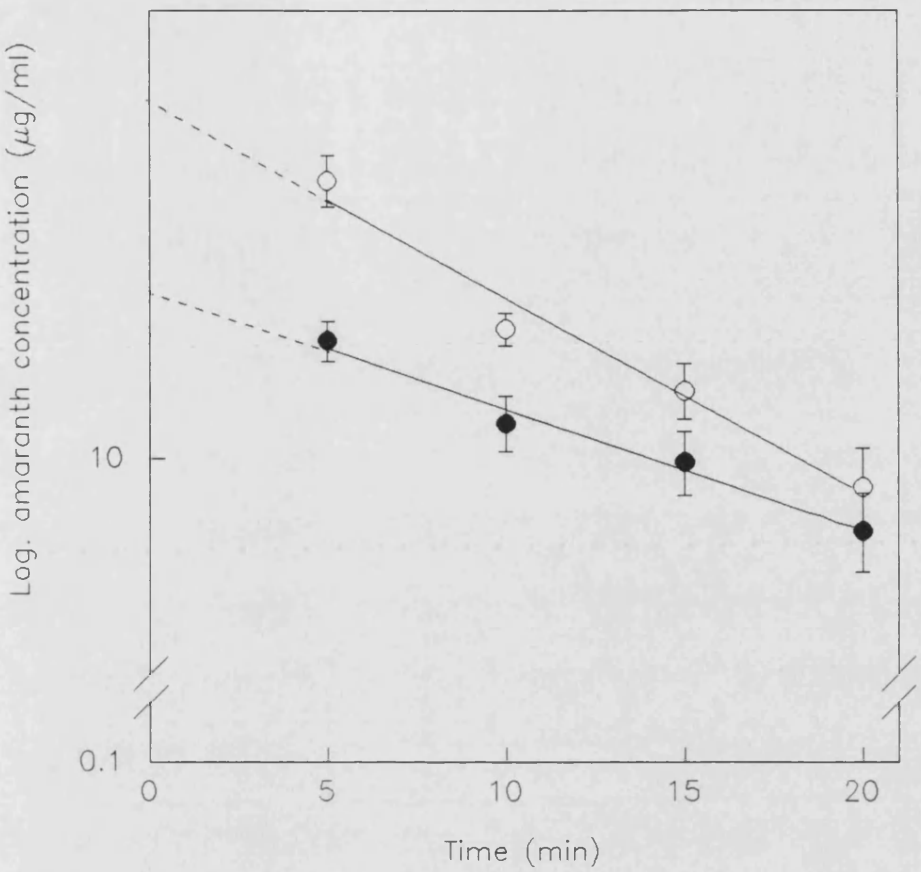


Figure 1. Concentration of amaranth in locust haemolymph over 20 minutes after injection of 25 μl of 2% amaranth in locust saline (○) or 2% amaranth plus 25 μg destruxin A in locust saline (●). Mean ± S.E. of six locusts in each case. Estimated mean value of amaranth concentration immediately after injection into (○) control locusts = 1.26 μg/ml and into (●) destruxin A treated locusts = 1.12 μg/ml.

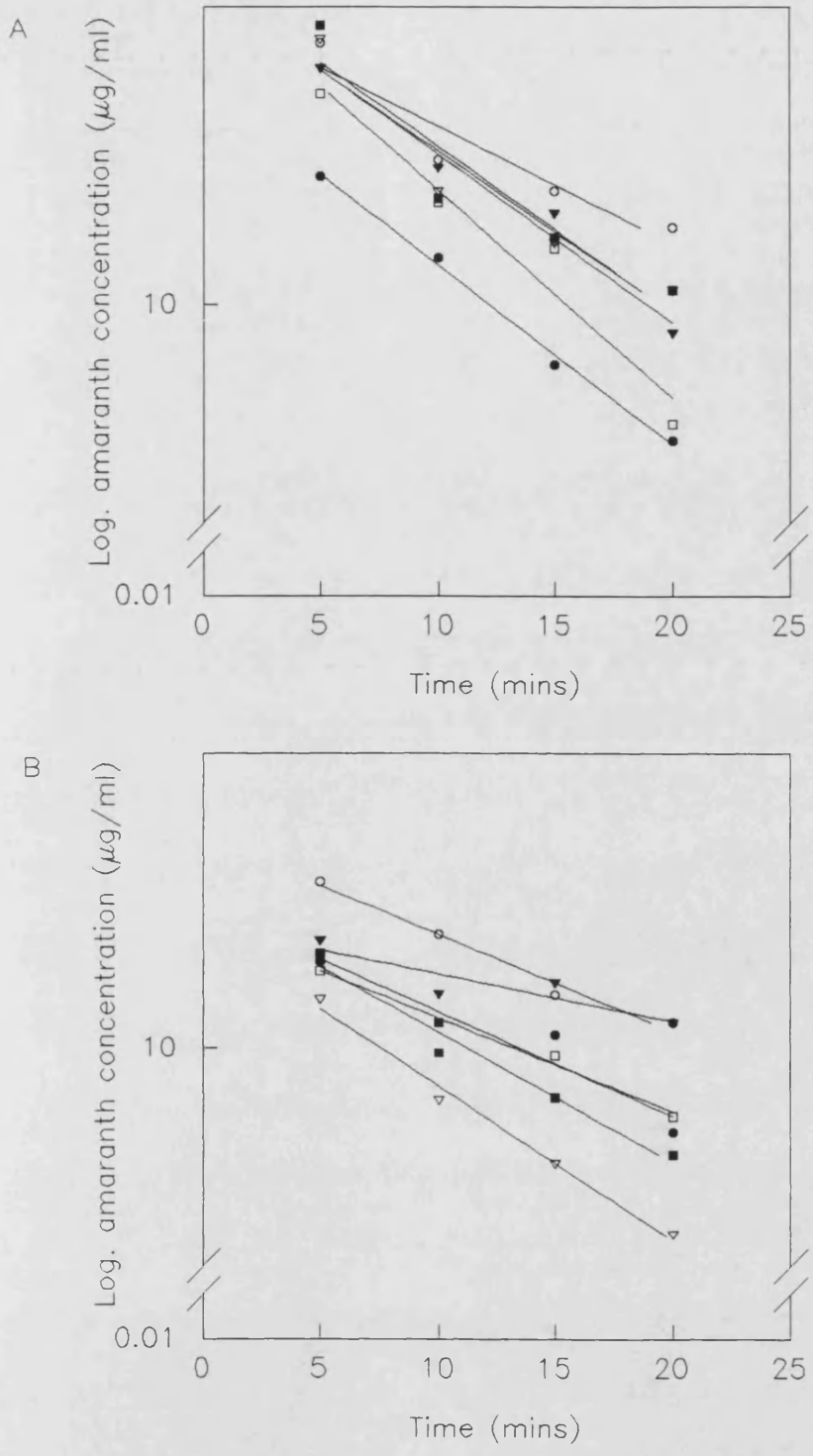


Figure 2. Concentration of amaranth in the haemolymph of individual locusts over the first 20 minutes after injection of 25 μl of (A) 2% amaranth in locust saline or (B) 2% amaranth plus 25 μg destruxin A in locust saline. Six locusts were used in each case. Regression lines have been drawn through the data.

infected locusts, the amaranth took much longer to circulate around the body than in the control locusts. This may have led to interference between the circulation of the amaranth around the body and the removal of the amaranth from the body. This would have produced an inaccuracy in any samples taken.

To test for any difference in circulation between control and infected locusts, 11 of each were injected with 25 µl of a 2% amaranth solution in locust saline, and observed every minute for 5 min. The spread of the dye within the locust was recorded after each minute. Table 1 shows the difference in distribution between control and infected locusts after 5 min.

It can be seen that the posterior distribution of the dye is little different between control and infected locusts. In almost all control ($^{10}/_{11}$) and infected ($^{9}/_{11}$) locusts the tip of the abdomen is pink after 5 min. In all the controls and a large number of the infected locusts ($^{7}/_{11}$), one or both of the metathoracic legs is pink to the mid-joint, after 5 min. The anterior distribution of the dye is quite different. After 5 min the pronotum and one or both of both the meso- and pro-thoracic legs are pink in almost all of the controls, but in only a third or less of the infected locusts.

Representative examples of the difference in amaranth distribution between control and infected locusts are shown in Figs. 3a and 3b. Fig. 3a is a control locust 5.5 min after amaranth injection, and it can be seen that the dye has reached the tip of the abdomen, the mid-joint of both metathoracic legs, and the tip of both the meso- and the pro-thoracic legs. The antennae are also pink. In comparison Fig. 3b shows an infected locust 5.5 min after amaranth injection. Again the tip of the abdomen and the mid-joint of both metathoracic legs are pink, but only the base of the mesothoracic legs and the neck are pink. The meso- and pro-thoracic legs themselves, and the antennae, are not pink.

To visualise more clearly the difference between control and infected locusts the locusts were scored individually. Each locust was given a score of 1 for each of the body parts, listed in Table 1 (except the pronotum), reached by the injected

Table 1. Distribution of amaranth to various parts of the locust body 5 min after injection into the third dorsal sclerite. 11 control locusts, inoculated with cotton seed oil, and 11 locusts inoculated with *Metarhizium anisopliae* isolate Me1, in cotton seed oil, were used on day 4 after inoculation.

Body part reached by amaranth after 5 min	**Number of controls (total = 11)**	**Number of infected (total = 11)**
Tip of abdomen	10	9
Mid-joint of 1 or both metathoracic legs	11	7
Pronotum	11	4
Tip of 1 or both mesothoracic legs	11	4
Tip of both mesothoracic legs	10	2
Tip of 1 or both prothoracic legs	10	1
Tip of both prothoracic legs	9	0

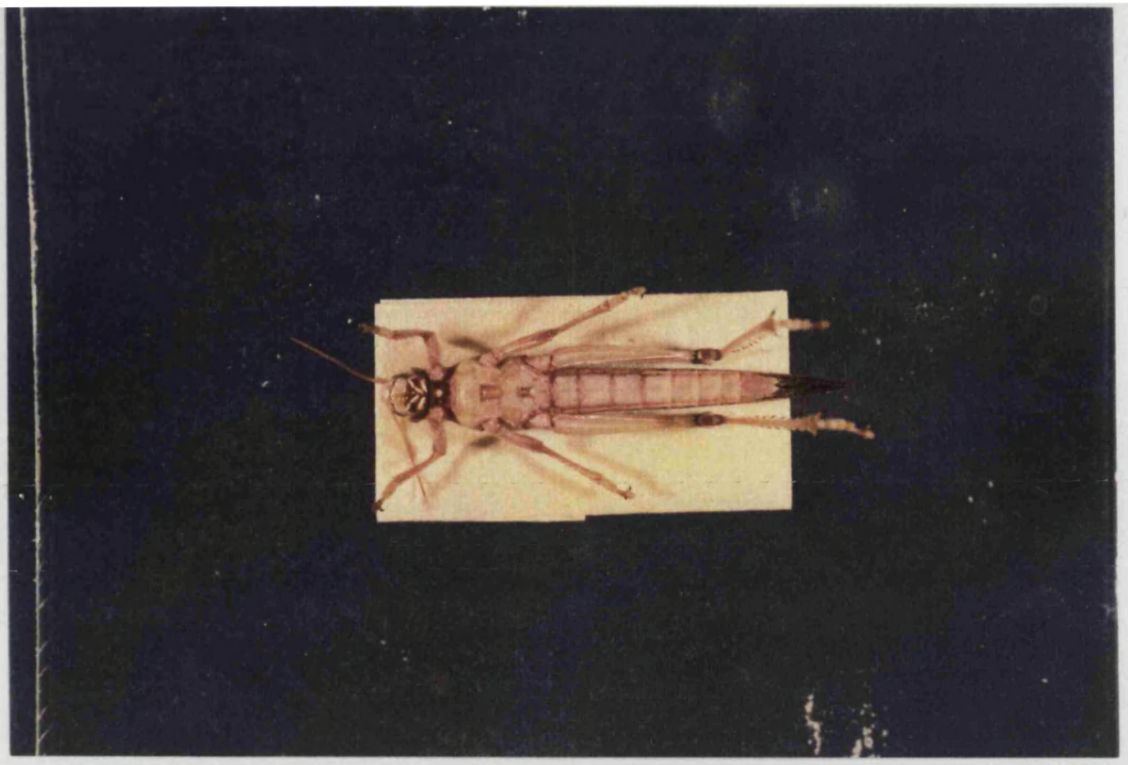


Figure 3a. Ventral surface of a control locust showing the distribution of amaranth 5.5 min after the injection of 25 µl of 2% amaranth in locust saline into the third dorsal sclerite.

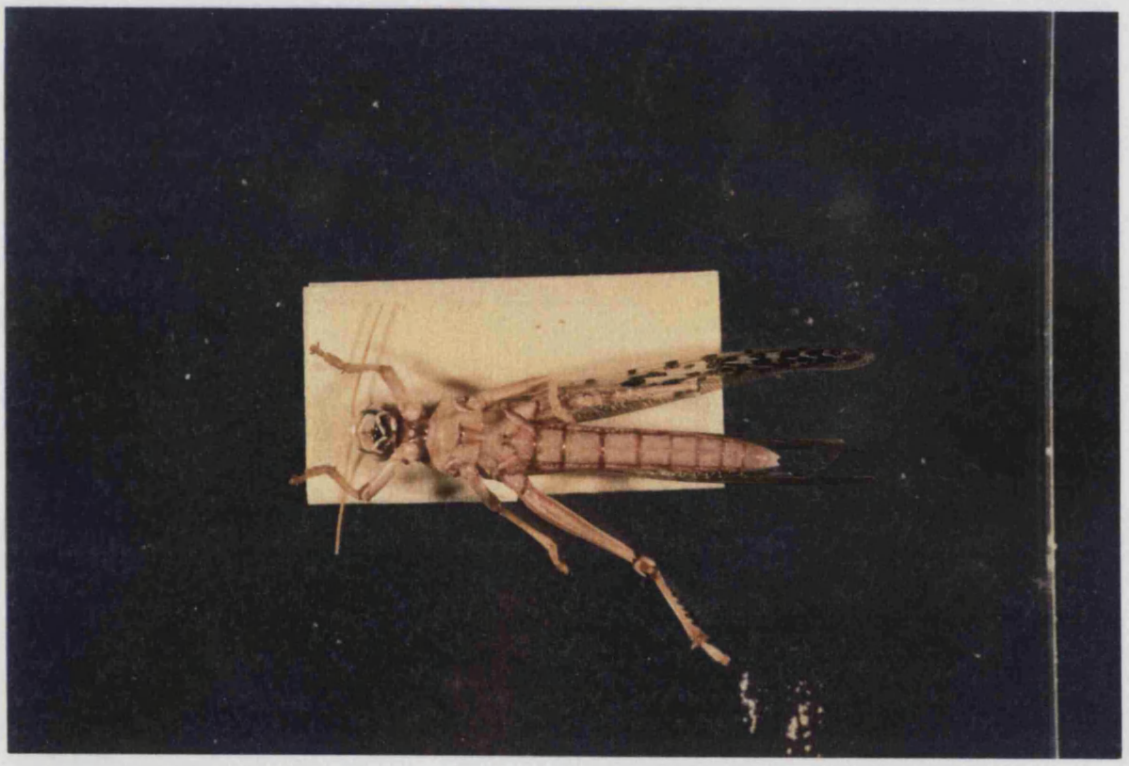


Figure 3b. Ventral surface of a locust infected with *M. anisopliae* isolate Me1 showing the distribution of amaranth 5.5 min after the injection of 25 µl of 2% amaranth in locust saline into the third dorsal sclerite.

amaranth after each minute. The cumulative mean (± standard error) was plotted against time and is shown in Fig. 4. Clearly, the distribution of amaranth within control locusts is significantly faster than in infected locusts.

Because the infected locusts were difficult to bleed and appeared to contain less haemolymph than the control locusts it was thought possible that the infected insects had a lower body-water content than controls. To test this, 10 control and 10 infected locusts were weighed on the day of inoculation and each subsequent day, up to and including day 4 (3 infected locusts were dead by day 4). The locusts were kept as before in individual containers, at 28°C, 30% RH with a 16 h light : 8 h dark cycle, with no access to food or water. The weights of the locusts are plotted in Fig. 5A and B. Regression lines were drawn through individual data sets, and from these the individual rates of water loss were calculated. It was found that there was no significant difference in the rate of water loss between control and infected locusts (T-test, $t = 0.96$, $p = 0.35$). The locusts were killed on day 4 and dried to constant weight. The water content was then calculated for each locust and it was found that there was no significant difference in water content between control and infected locusts, control locusts mean water content = 0.521g ± 0.024 (59.05% ± 0.39 of wet weight on day 4) and infected locusts mean water content = 0.570g ± 0.018 (58.61% ± 0.82 of wet weight on day 4) (T-test, $t = -0.76$, $p = 0.46$).

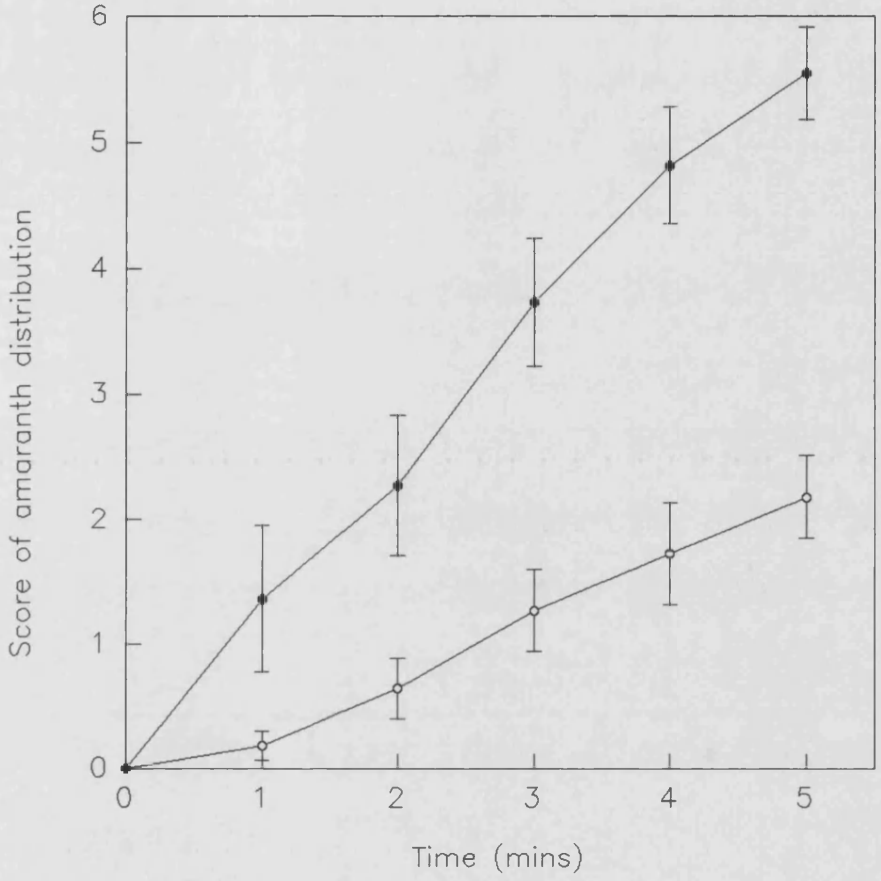


Figure 4. Cumulative score of amaranth distribution within individual control locusts (•) and locusts infected with Me1 (∘) over the first 5 min after injection of 25 μl of 2% amaranth in locust saline. Mean ± SE. n = 11

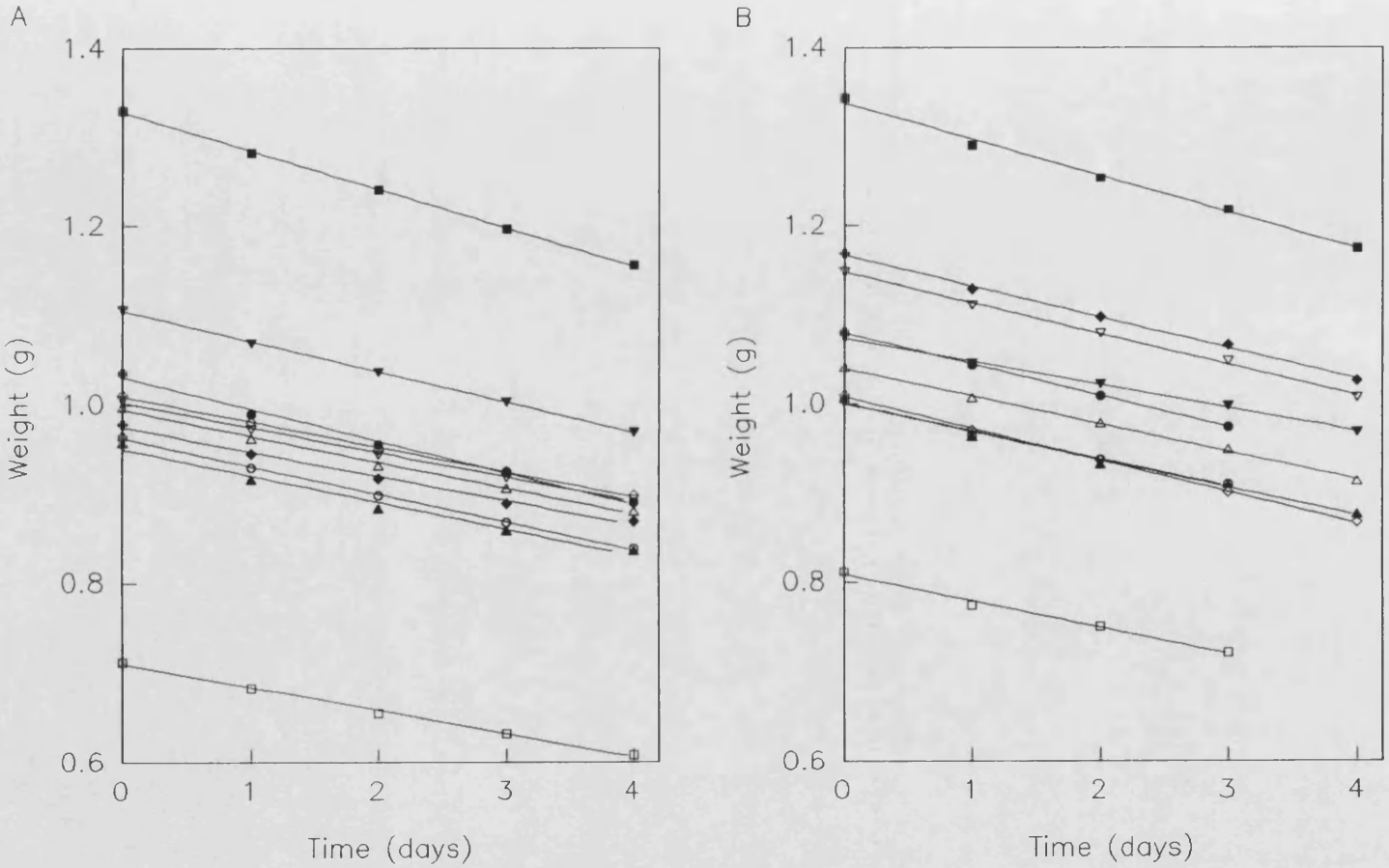


Figure 5. Wet weight of (A) control and (B) Me1 infected locusts over 4 days. The locusts were denied access to food or water over this period. Regression lines have been drawn through individual data sets. 10 locusts used in each case.

DISCUSSION

Destruxin A is seen to have an inhibitory effect on the rate of removal of injected amaranth from locust haemolymph. This effect is likely to be due to the action of destruxin on the Malpighian tubules, as the Malpighian tubules are responsible for the removal of the dye from the haemolymph (Maddrell *et al.*, 1974). Destruxin A is known to inhibit Malpighian tubule fluid secretion *in vitro* (see chapter 3; James *et al.*, 1993) and the results shown here indicate that destruxin A is able to inhibit the transport of amaranth by locust Malpighian tubules *in vivo*.

Destruxin A may also have inhibited fluid transport *in vivo*, although the evidence is less direct. Since equal amounts of dye solution were injected into control and destruxin-treated locusts, it would be expected that the initial dilution of the dye would have been similar. It is clear from Fig. 1, however, that this was not the case, the lines for control locusts projecting back to a value at zero time that is considerably greater than for the destruxin-injected locusts. This implies that the blood volume of the treated locusts was greater than that of the controls. Of course at the moment of injection (t=0) the blood volumes of the two groups of locusts must have been the same. An explanation for this observation that is consistent with other findings reported in this study (see chapter 3; James *et al.*, 1993) is that the larger apparent blood volume of the destruxin-treated insects was a consequence of the inhibition of urine formation by the Malpighian tubules.

As in other insects the circulation in locusts is an open system, with a contractile dorsal vessel that opens into the haemocoel at the head of the locust and ends blindly in the 10th abdominal segment. The haemolymph is pumped forward by the dorsal vessel into the haemocoel, where the resulting increase in pressure travels down the body so leading to blood being forced into the dorsal vessel through incurrent ostia (Chapman, 1982). In Acridoids the only other accessory pulsatile organs known are the antennal ampullae which force haemolymph into the antennae (Uvarov, 1966).

The pattern of amaranth distribution seen in the infected locusts is consistent with a slowed but normal circulation. The dye is injected into the haemocoel, filling the abdomen, and so reaching the base of the metathoracic legs, which, along with the abdomen, turn pink first. The amaranth is then carried backwards in the haemolymph before being taken into the dorsal vessel and pumped forward, thus making the fore-limbs, pronotum and antennae the last to become pink.

The casual observation that the infected locusts were difficult to bleed suggests a reduced blood volume. Although the circulation in infected locusts is seen to be much slower than in control locusts this is not due to any alteration in whole body water content, but it remains possible that there is a difference in the distribution of water between the two groups. The infected locusts could have more water in their tissues, or the amount of water utilised by the fungus could be sufficient to account for the difference.

Another explanation is that the circulation is reduced due to the presence of fungal matter in the blood. Although Samuels *et al.* (1988a) and Kershaw (1993) found few hyphal bodies in the haemolymph of Mel infected *M. sexta* larvae, Kershaw (1993) found a large number of blastospores, which Samuels *et al.* (1988a) did not mention. There may have been a large number of blastospores in the haemolymph of the Mel infected locusts, thus slowing down the circulation. This may also have made it difficult to collect blood.

Destruxins are known to increase both the amplitude and frequency of *M. sexta* larva heart beat at very low concentrations, but at high concentrations the effects of destruxins are inhibitory (Samuels , 1986). The concentration of destruxins in Mel infected *M. sexta* was seen to be low (0.018 μg/ml) and a similar if not lower level might be expected in Mel infected *S. gregaria* (see chapter 6). This might then lead to a stimulation of the dorsal vessel and so the reverse situation would be seen, however, in locusts, injected destruxin is seen to accumulate in the pericardial tissue (Cherton *et al.*, 1991), and this may lead to a localised high concentration, causing inhibition of the dorsal vessel. Prolonged exposure of the dorsal vessel to destruxins,

albeit low concentrations, perhaps during Me1 infection, could also lead to inhibition, and this may have occurred here.

CHAPTER 5. EFFECT OF DESTRUXIN A ON *SCHISTOCERCA GREGARIA* MALPIGHIAN TUBULE ULTRASTRUCTURE *IN VITRO.*

INTRODUCTION

The acute toxicity of destruxins to insect species varies according to the species tested, Lepidoptera and Diptera being particularly susceptible (Roberts, 1981; Samuels *et al.*, 1988a). Destruxins are known to cause physiological and ultrastructural changes to a range of tissues and cells from various insect species (Quiot *et al.*, 1985; Kershaw, 1993) and these cells respond differently to the destruxins, again reflecting variability in susceptibility (Quiot *et al.*, 1985)

The ultrastructure of larval *G. mellonella* (Vey and Quiot, 1989) and adult *C. vomitoria* (S.R. Watkins, J.S. Cook and A.K. Charnley, unpublished) Malpighian tubules has been seen to be altered by destruxins. The toxins caused extensive vacuolisation of the cytoplasm, swelling of the mitochondria and the breakdown of the basal infolds of the tubule cells.

The results of previous sections show that destruxins inhibit *S. gregaria* Malpighian tubule function both *in vitro* (see chapter 3; James *et al.*, 1993) and *in vivo* (see chapter 4). The Malpighian tubules are part of the insect's excretory system, and are thought to be involved in the removal of destruxins from the insect's body (Cherton *et al.*, 1991; Loutelier *et al.*, 1994), thus any effects of destruxins on the ultrastructure of the tubules would be important. In this chapter the possibility that the physiological effects of destruxins on *S. gregaria* Malpighian tubules seen earlier may be reflected by ultrastructural changes is investigated.

MATERIALS AND METHODS

Freshly fed adult female *S. gregaria* were used, between 4 and 7 days after ecdysis. The gut and associated Malpighian tubules were removed, with the head intact, and the end of the gut ligatured to avoid any gut contents contaminating the preparation. The whole of the gut except the head was then incubated in saline or saline plus 100 μM destruxin A at 28°C. A control was taken at 0 min, and subsequent control and destruxin treated tubules were collected after 9, 19, 60 and 180 min incubation. Tubules were removed from two separate locusts at each incubation time, for each treatment. Between 3 and 7 tubules were observed per locust, thus 6 to 14 tubules, each with 2 to 4 cells, were examined for each treatment. The tubules were fixed in 2% gluteraldehyde in half strength locust saline (pH 7.2) for 4 h, and washed in full strength locust saline (pH 7.2) for 15 min. Tubules were then post fixed in 1% osmium tetroxide in full strength locust saline (pH 7.2) for 1 h before a final wash in distilled water. Tubules were dehydrated in a graded series of acetones (50%, 70%, 80%, 90%, 95%, 100%, and 100% dry) and embedded in TAAB epoxy resin. Thin sections were cut using an ultramicrotome (Reichert OMU3, Leica, UK Ltd.) then stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Sections were examined under a JEOL 1200EX transmission electron microscope operated at 80KV.

RESULTS

Fig. 1 shows a section through the wall of a tubule from a 0 min control, showing it to be only one cell thick. The main features of this, the predominant cell type of *S. gregaria* Malpighian tubules, can be seen. According to the terminology of Charnley (1982) and Garrett *et al.* (1988) these cells are referred to as primary or type 1 cells. A basal lamina surrounds the basal surface of the cell. The basal membrane within this is highly folded, with very few mitochondria or vacuoles between the folds. Conversely the cytoplasm contains a large number of vacuoles and mitochondria and the nucleus, which is round in cross-section, is clearly visible. The apical surface is formed into many microvilli which extend into the lumen of the tubule. Mitochondria can be seen inside these microvilli.

A small number of profiles of a different cell type were seen, possibly a type 2 cells or mucocyte. One of these from a 0 min control is shown in Figs. 2a and b. The cell is seen to contain a profusion of clear, lightly stained vesicles which are very large at the apical side of the cell, basal membrane infoldings that do not penetrate far into the cell, very few mitochondria, few, thin microvilli, and no mitochondria within these microvilli. Endoplasmic reticulum and Golgi bodies are also more evident than in the type 1 cells (Fig. 2a).

A comparison was made between control and treated tubules of three main features of the type 1 cells, the basal membrane infoldings, the microvilli and the mitochondria. The basal membrane infoldings in Fig. 3a, a control tubule at 0 min, are regular and even, with some mitochondria between them but very few vacuoles. The mitochondria themselves are no greater than 200 nm in width, with their cristae obvious (Fig. 3b). At the apical surface the microvilli are long and even with long, thin mitochondria within them (Fig. 3c).

After 9 min the cells of both the destruxin treated and the control tubules appear no different from those of the 0 min control (results not shown). After 19 min the microvilli, in both the control and treated tubules, appear thin and regular with thin mitochondria within them (Figs. 4a and b). In general these and the cytoplasmic

Figure 1. Transmission electron micrograph of a section through the wall of a *S. gregaria* Malpighian tubule. The tubule is one cell thick, with a basal lamina (A) surrounding the basal surface. Basal membrane infoldings (B) are clearly visible within this. The cytoplasm contains a nucleus (N), many mitochondria (M) and vacuoles (V). The apical surface is formed into many microvilli (R), projecting into the lumen (L). Bar = 1 μm.

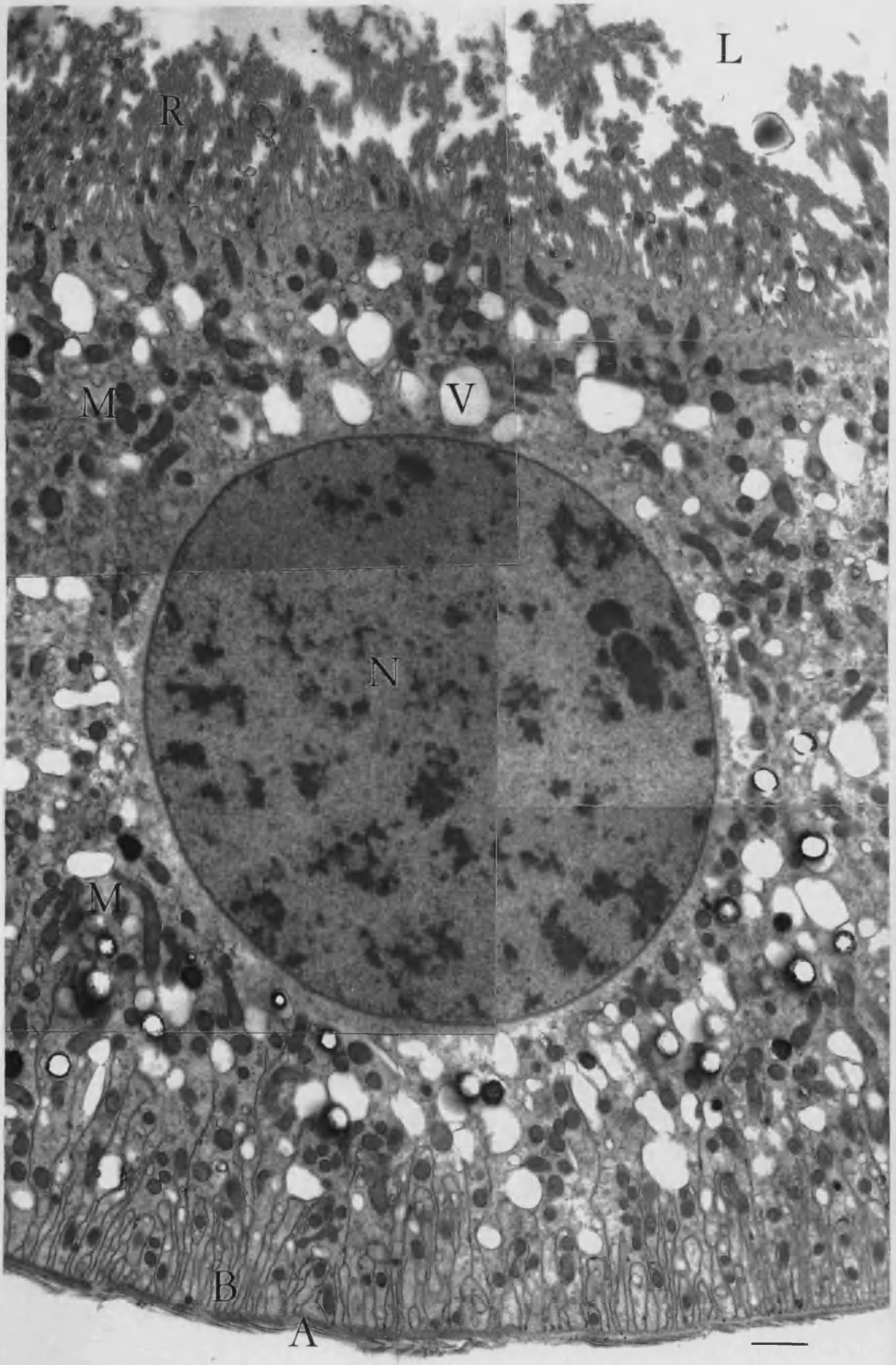
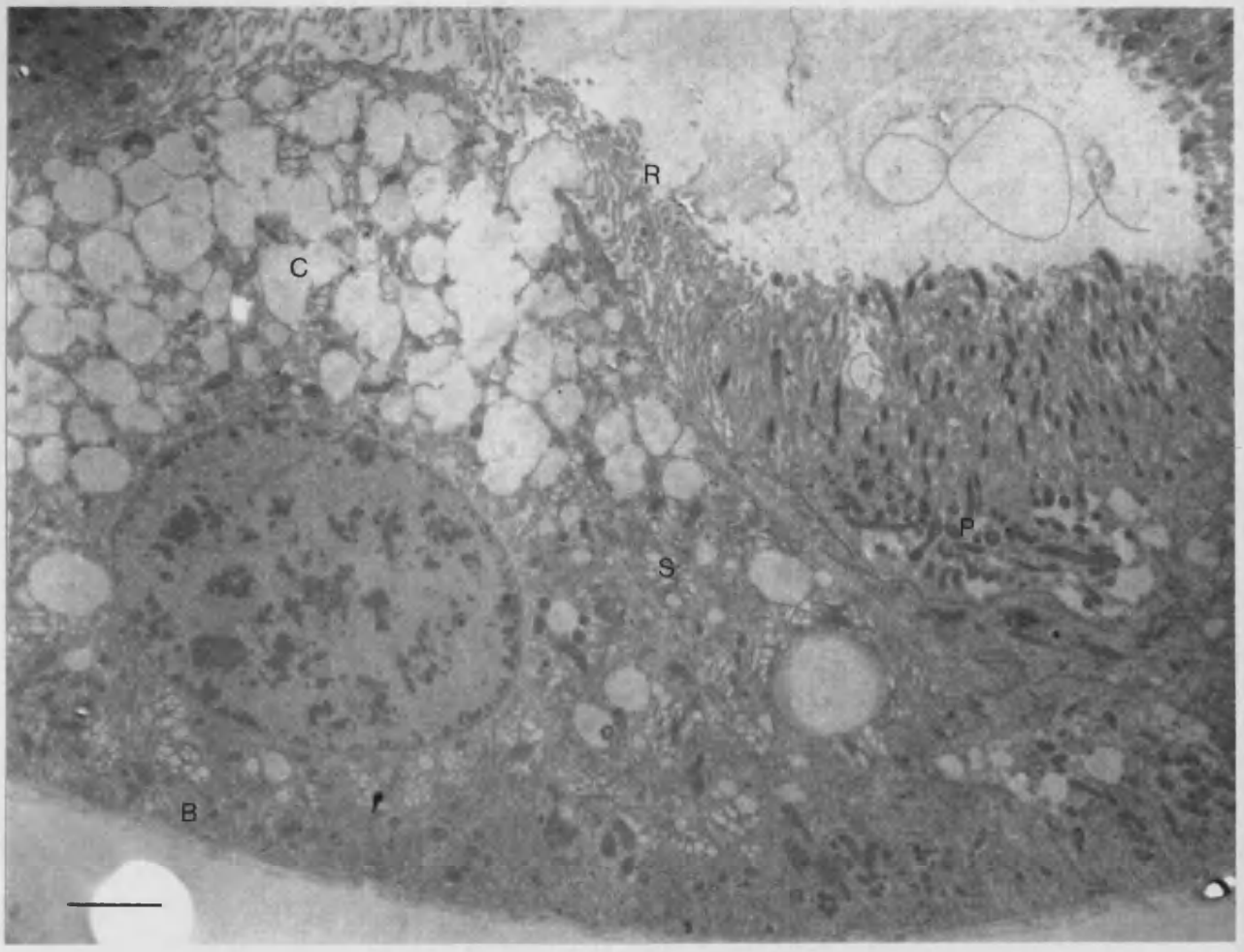

L
R
M
V
N
M
B
A

Figure 2a. Transmission electron micrograph of a second cell type (S) bordering on a primary cell (P). The second cell type has fewer , thinner microvilli (R) than the primary cell, has many vesicles (C), and basal membrane infoldings (B) that do not penetrate far into the cell. Bar = 2 μm.

Figure 2b. Golgi bodies (G) and endoplasmic reticulum (E) are prominent in the second cell type. Bar = 1 μm.


R
C
P
S
B

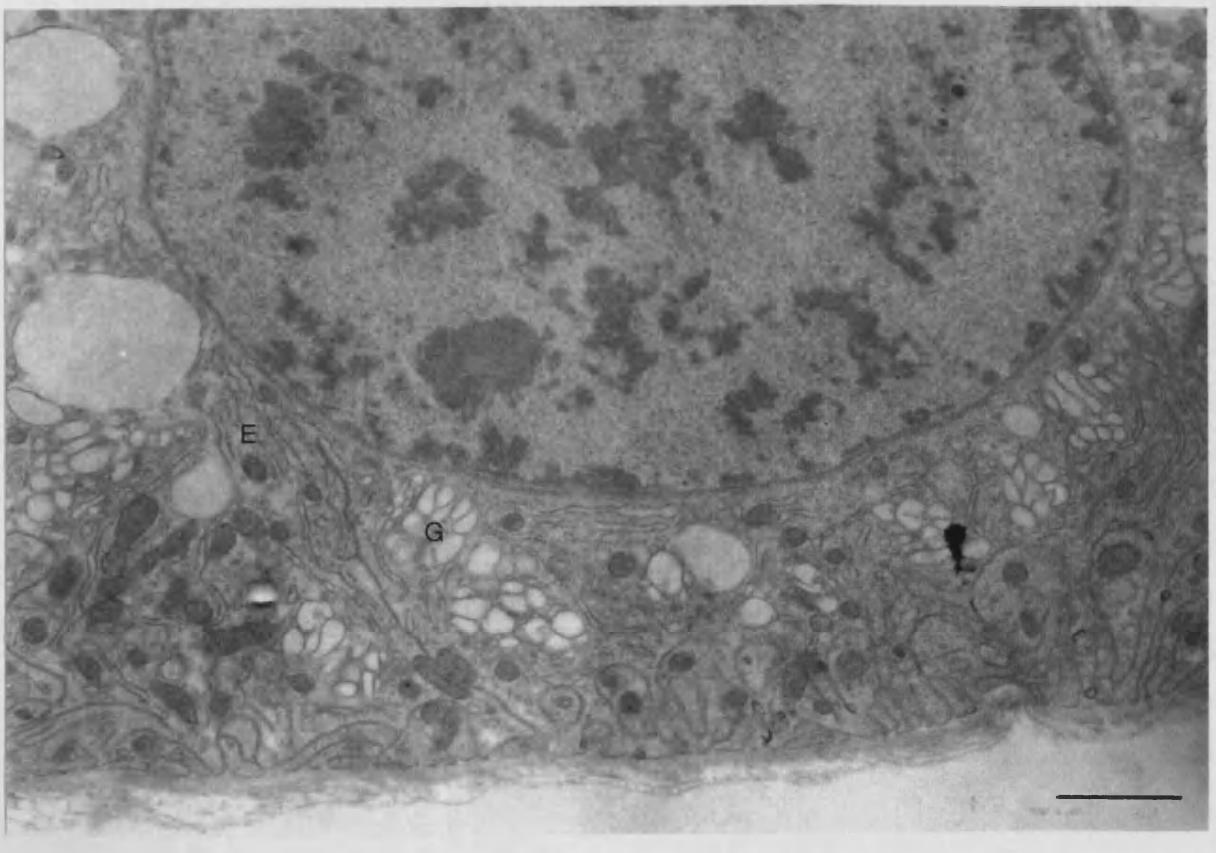
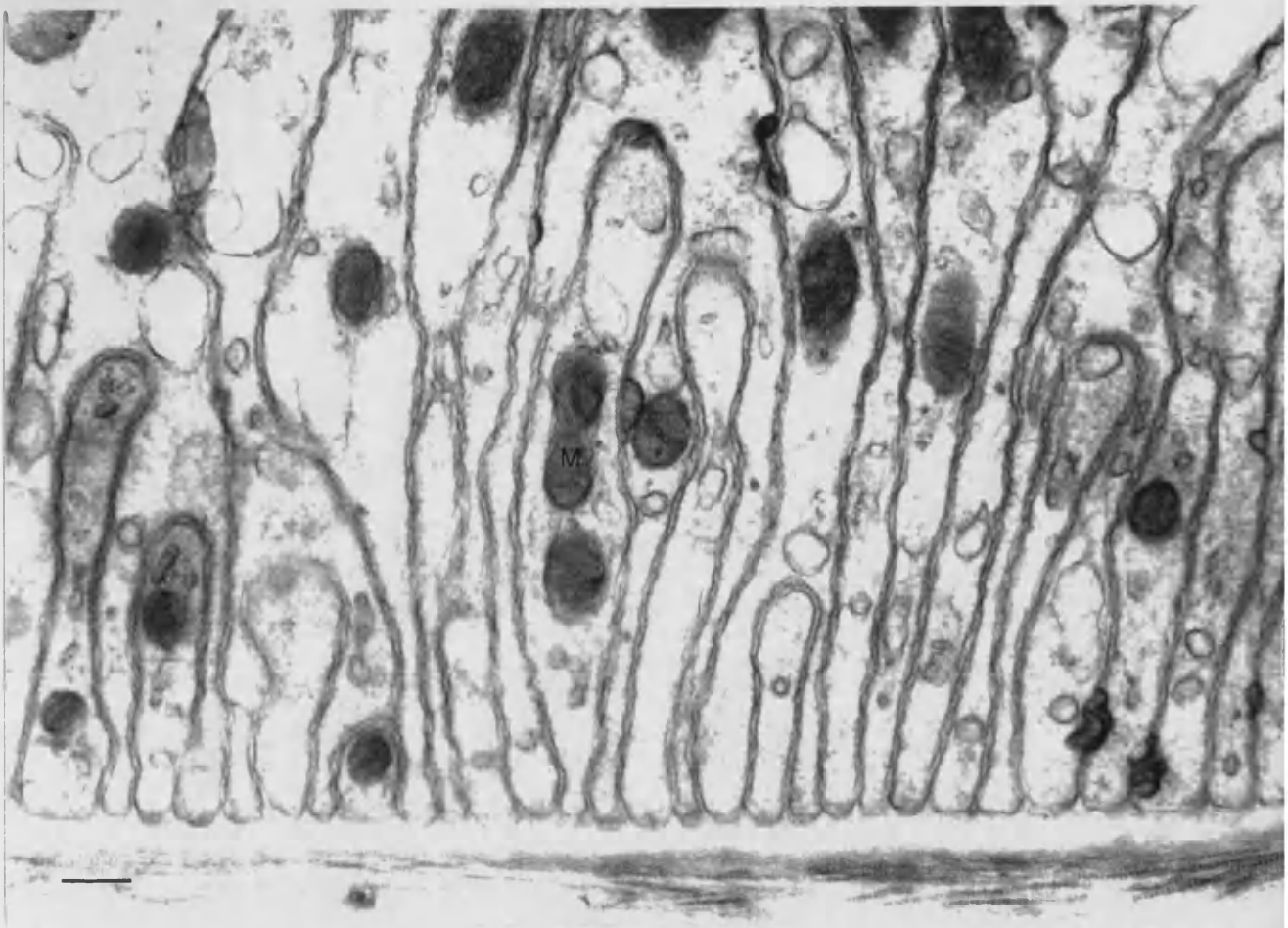

E
G

Figure 3a. The basal membrane infoldings of a control cell (0 min) showing their regular, even arrangement and few mitochondria (M) within them. Bar = 200 nm.

Figure 3b. Mitochondria within the cytoplasm of a control cell (0 min). Bar = 500 nm.


M

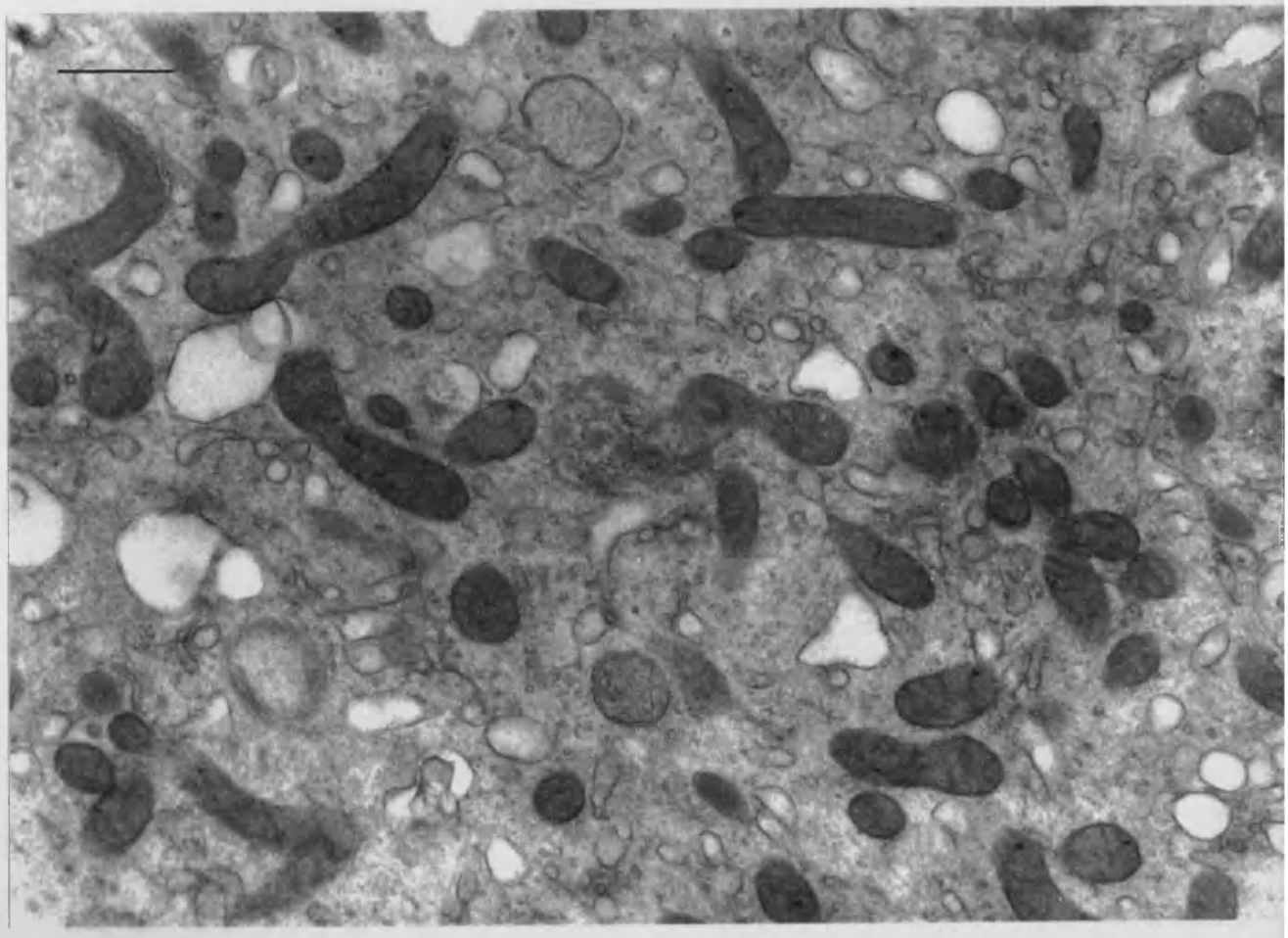
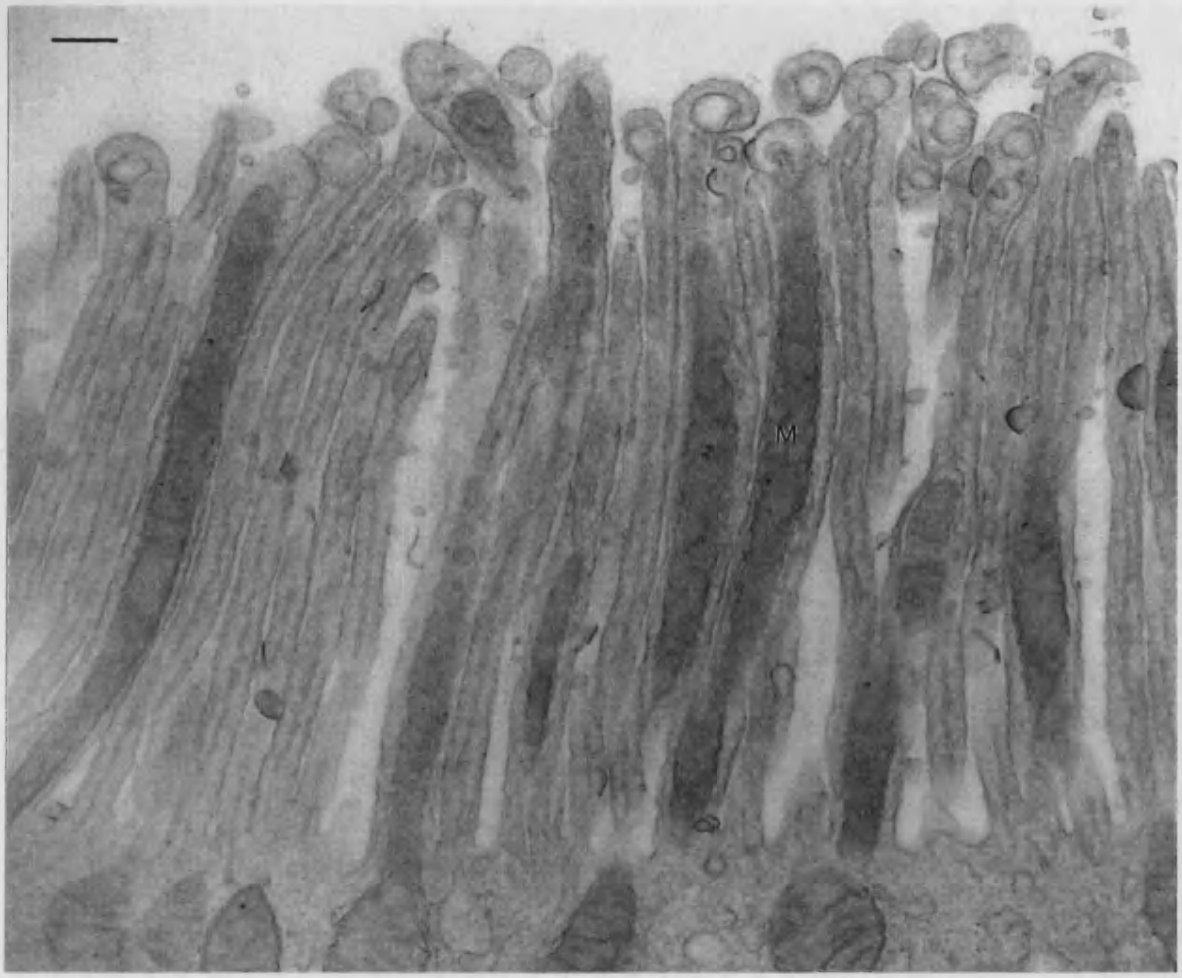


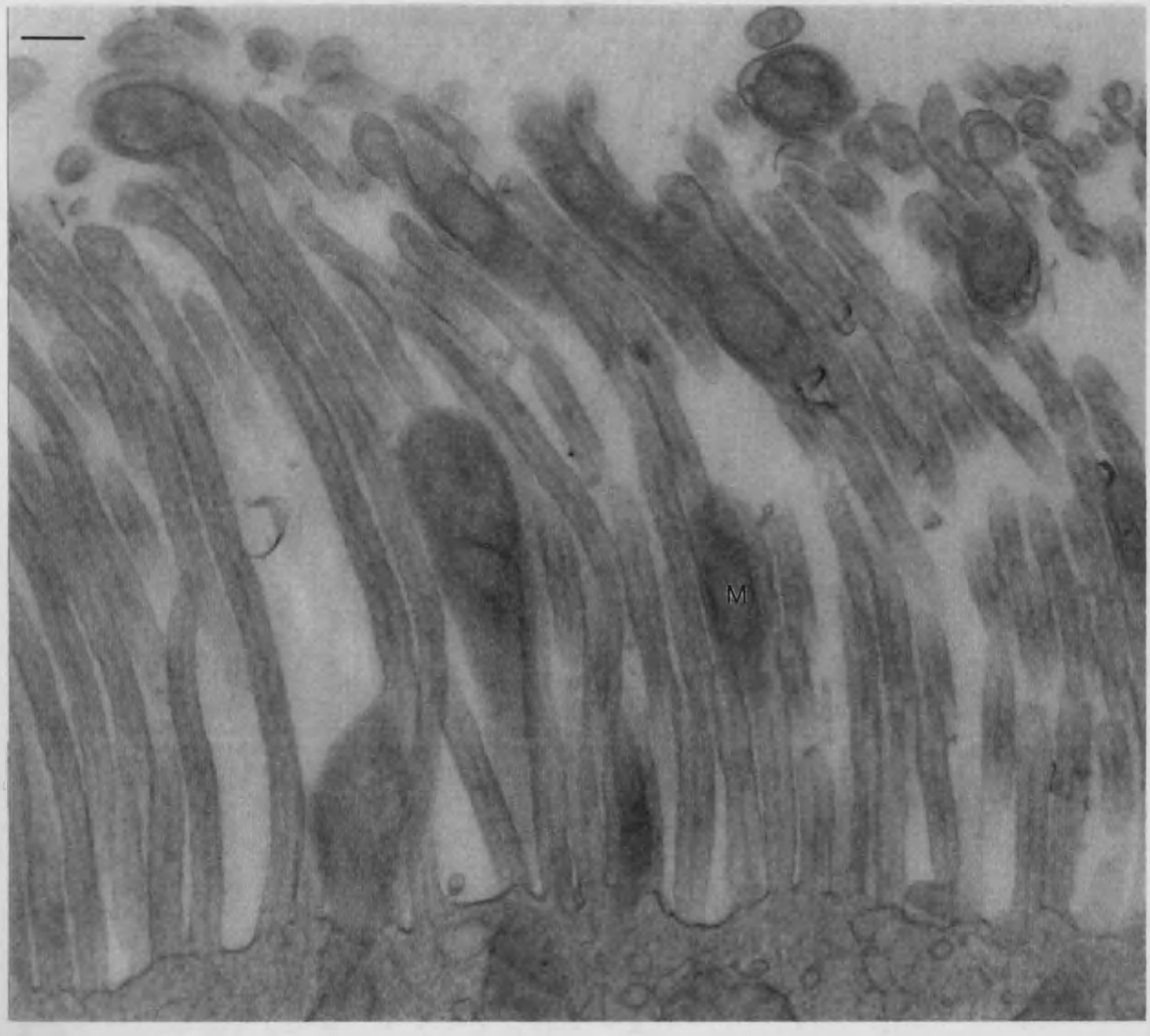
Figure 3c. Thin, even microvilli of a control cell (0 min) with thin mitochondria (M) within them. Bar = 200 nm.


M

Figure 4a. Thin, even microvilli of a control cell (19 min) with thin mitochondria (M) within them. Bar = 200 nm.

Figure 4b. Thin, even microvilli of a destruxin treated cell (19 min) with thin mitochondria (M) within them. Bar = 200 nm.


M


M

mitochondria are 200 nm in width (Figs. 4c and d). These features, in both treatments, and the basal membrane infoldings of the control tubules (Fig. 4e), are no different from the 0 min control. However, a small number of the destruxin treated tubule cells appear to be losing the regular, parallel structure of their basal membrane infoldings, and there are a large number of vacuoles within the infolds (Fig. 4f).

Figs. 5, 6 and 7 show sections of control and destruxin treated tubule cells after 1 h. There are obvious differences between these and the 0 min controls, but fewer differences between the 1 h destruxin treated tubules and the 1 h controls. The most notable feature of both is the mitochondria which appear swollen. Many in the 1 h control are little larger than in the 0 min control, with few greater than 400 nm in width and most retaining their original shapes (Fig. 5a and 5b). In the destruxin treated tubules, however, a large number have swollen to spheres and are up to 600 nm in width (Fig. 5c). The cristae are clearly visible in the mitochondria of both treatments but appear as fragments attached to the outer membrane.

The basal membrane infoldings of the 1 h destruxin treated tubule cells differ markedly from the 0 min controls. In all the cells, the infolds contain vacuoles (Fig. 6a) and in over half of the cells the infolds themselves have begun to breakdown, pinching off to form what appear to be vesicles at the ends of the infolds (Fig. 6b). By contrast, over half of the 1 h control tubule cells have regular, even basal membrane infoldings (Fig. 6c) just as those in the 0 min controls. Only about a third of the 1 h control cells have the same degree of vacuolisation exhibited by the 1 h destruxin treated tubules (Fig. 6d) and vesicle formation at the inner edge of the basal infolds was seen only rarely.

Generally, the apical microvilli in the 1 h control tubules appear similar to those in the 0 min controls. They are regular and thin, with thin mitochondria, although some mitochondria are swollen making the microvilli appear distended (Fig. 7a). This swelling is more pronounced in the destruxin treated tubules. In a small number of 1 h destruxin treated tubules the microvilli are fragmented, with pieces in

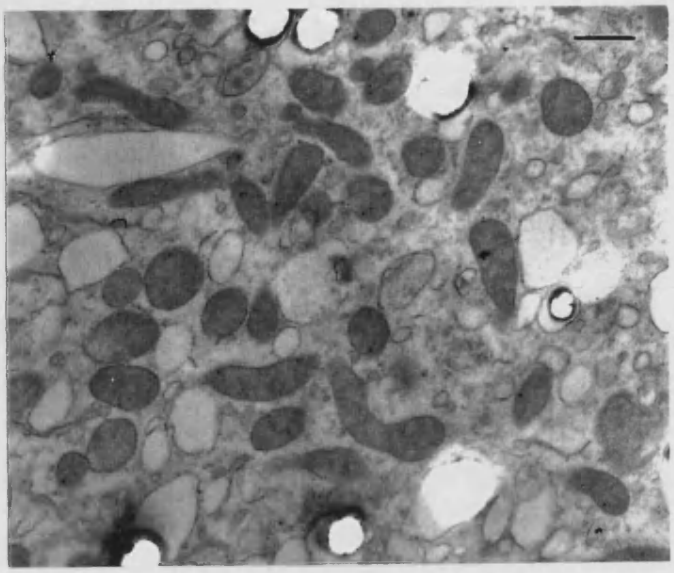


Figure 4c. Mitochondria within the cytoplasm of a control cell (19 min) which appear normal. Bar = 500 nm.

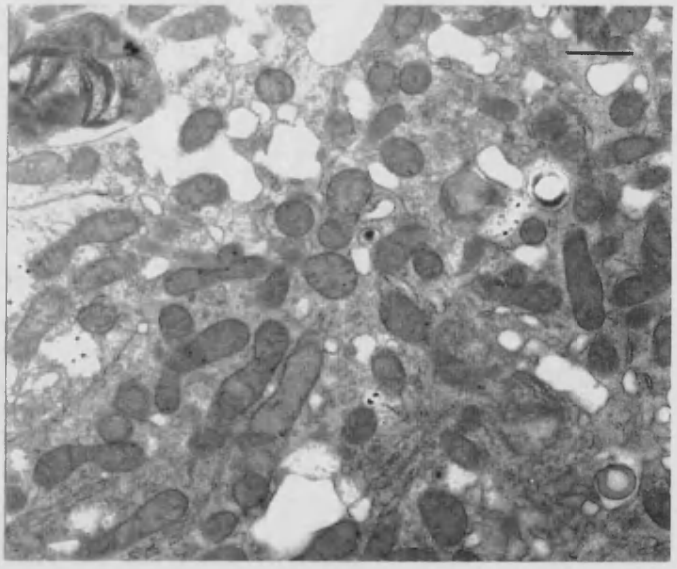
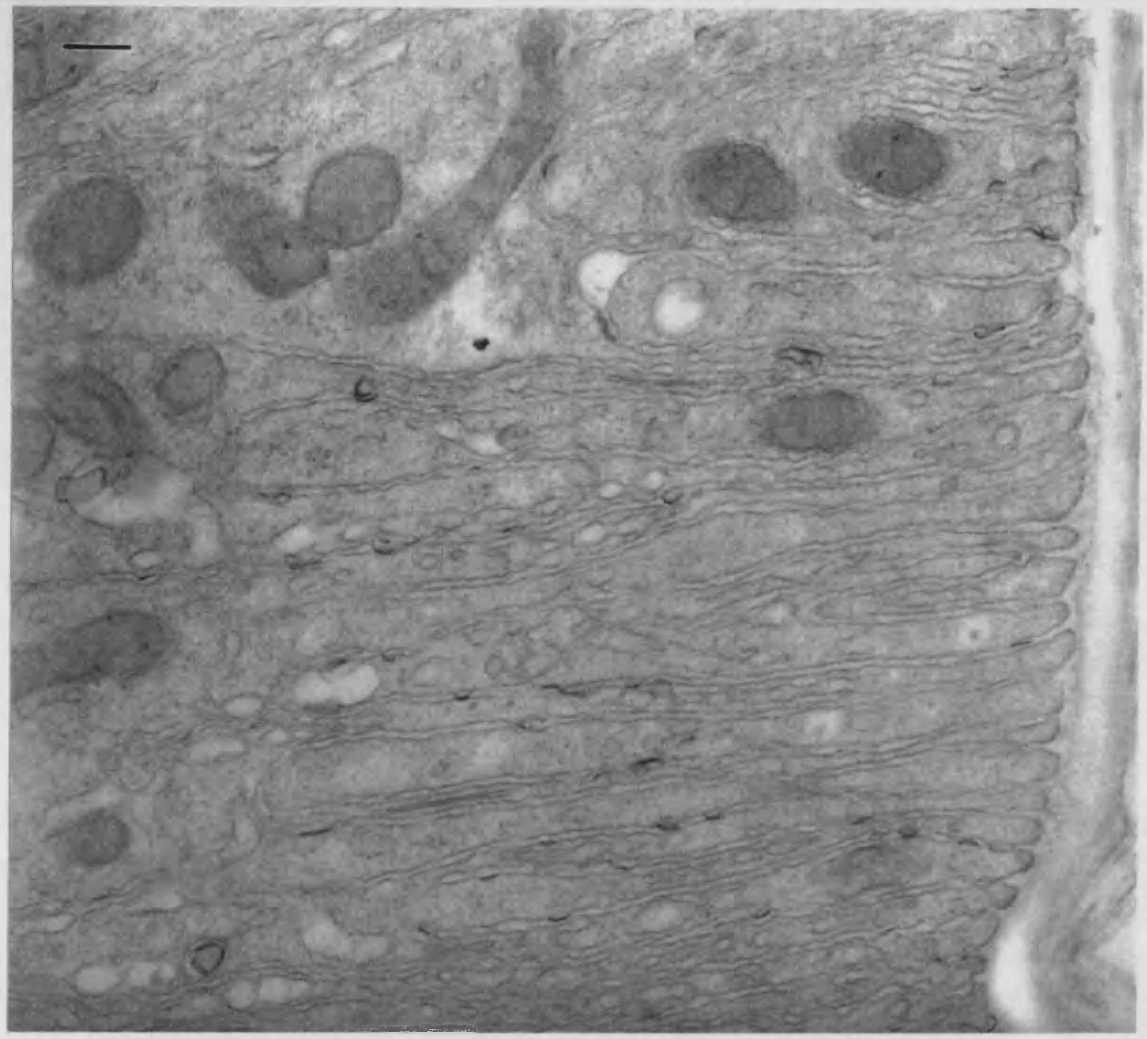


Figure 4d. Mitochondria within the cytoplasm of a destruxin treated cell (19 min) which appear normal. Bar = 500 nm.

Figure 4e. Regular and even basal membrane infoldings from a control cell (19 min), with few mitochondria or vacuoles within the infolds. Bar = 200 nm.

Figure 4f. The basal membrane infoldings of a destruxin treated cell (19 min). There are a number of vacuoles (V) within the infolds and the infolds themselves are losing their regular appearance. Bar = 500 nm.



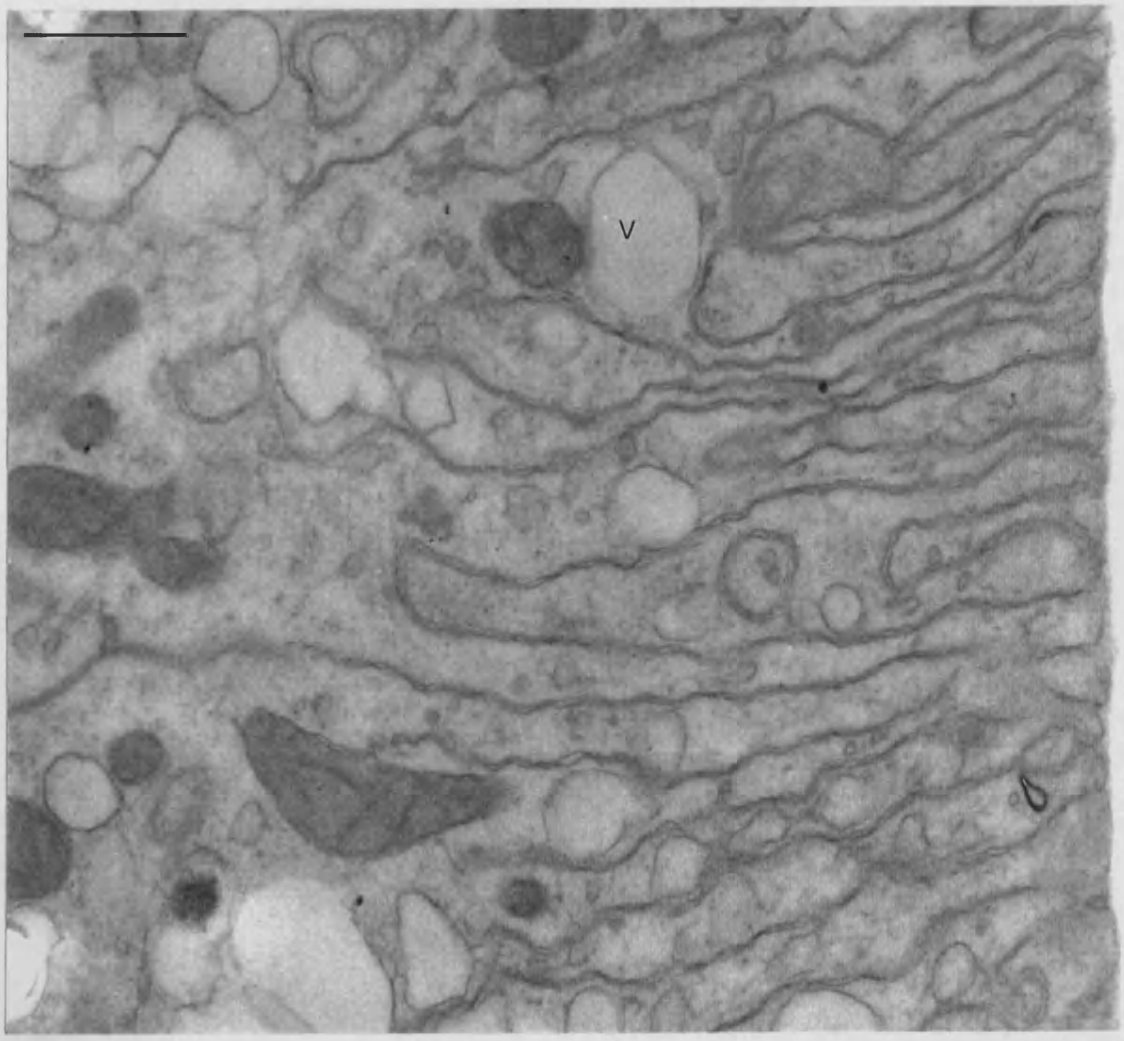
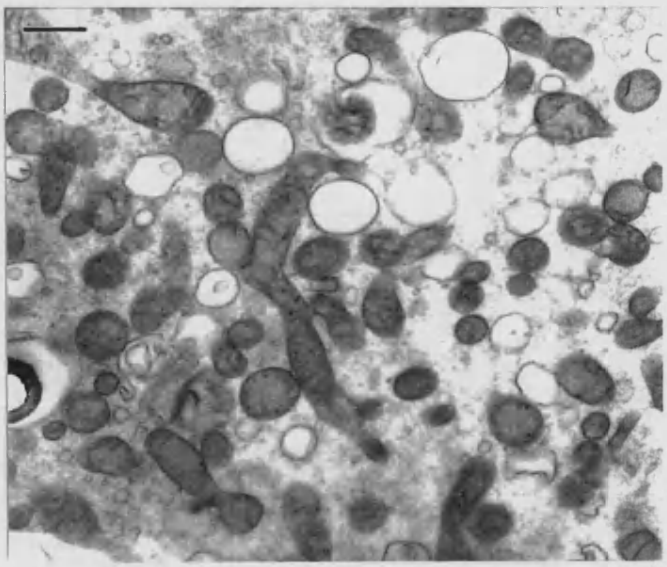
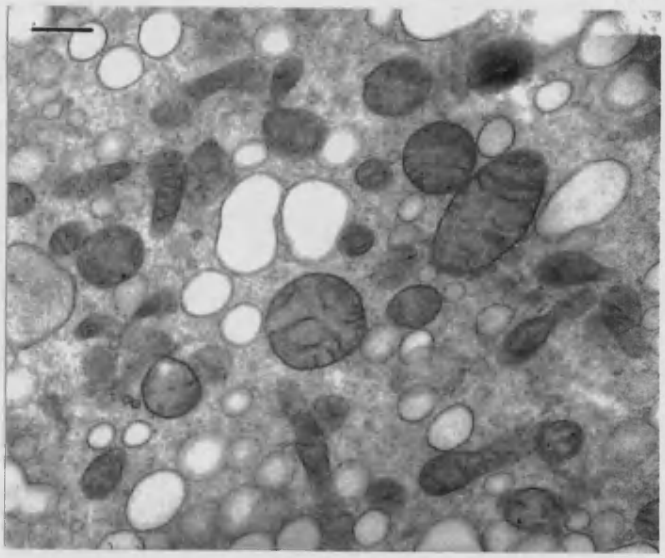

V

Figure 5a. Mitochondria within the cytoplasm of a control cell (1 h) which appear normal. Bar = 500 nm.

Figure 5b. Mitochondria within the cytoplasm of a control cell (1 h) which appear swollen. The cristae are only fragments attached to the outer membrane. Bar = 500 nm.

Figure 5c. Greatly swollen mitochondria within the cytoplasm of a destruxin treated cell (1 h). The cristae are clearly visible as fragments attached to the outer membrane. Bar = 500 nm.





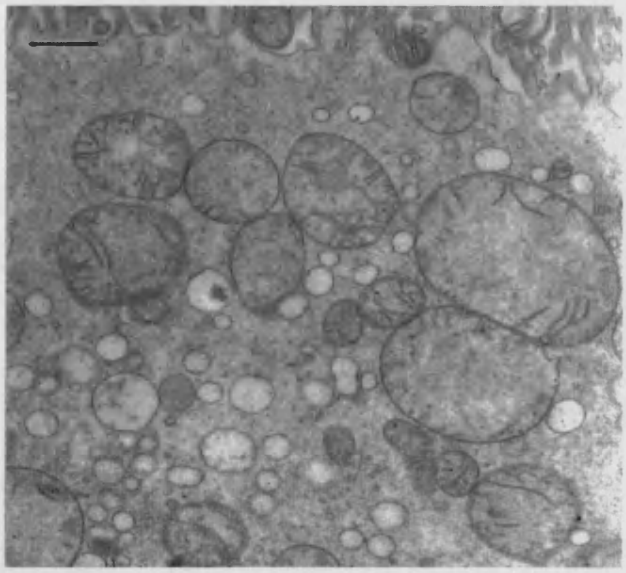
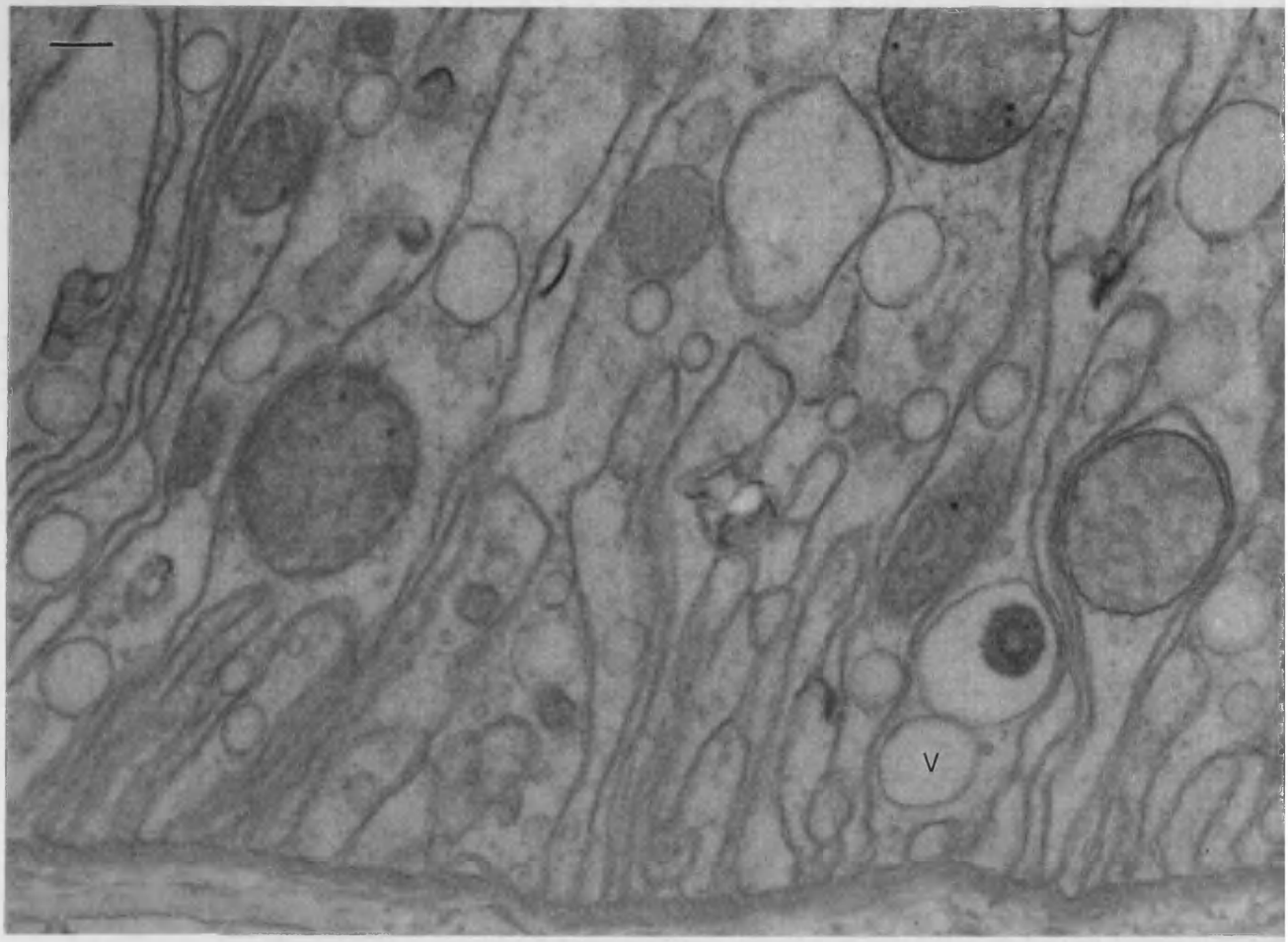


Figure 6a. The basal membrane infoldings of a destruxin treated cell (1 h) showing many vacuoles (V) within the infolds. Bar = 200 nm.

Figure 6b. The basal membrane infoldings of a destruxin treated cell (1 h). The infolds have broken down and fragmented, forming vesicles at the ends of the infolds (X). Bar = 200 nm.


V

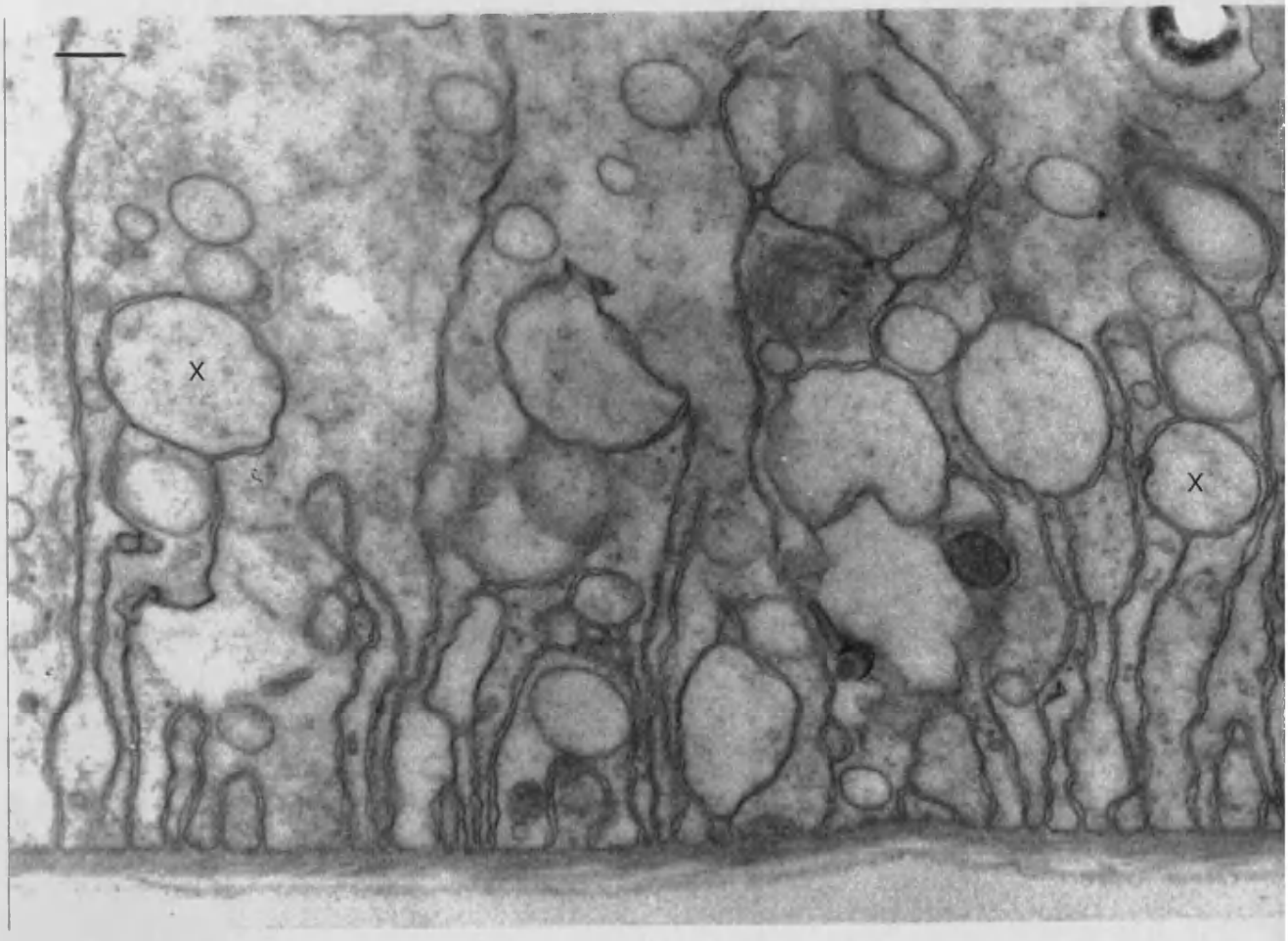
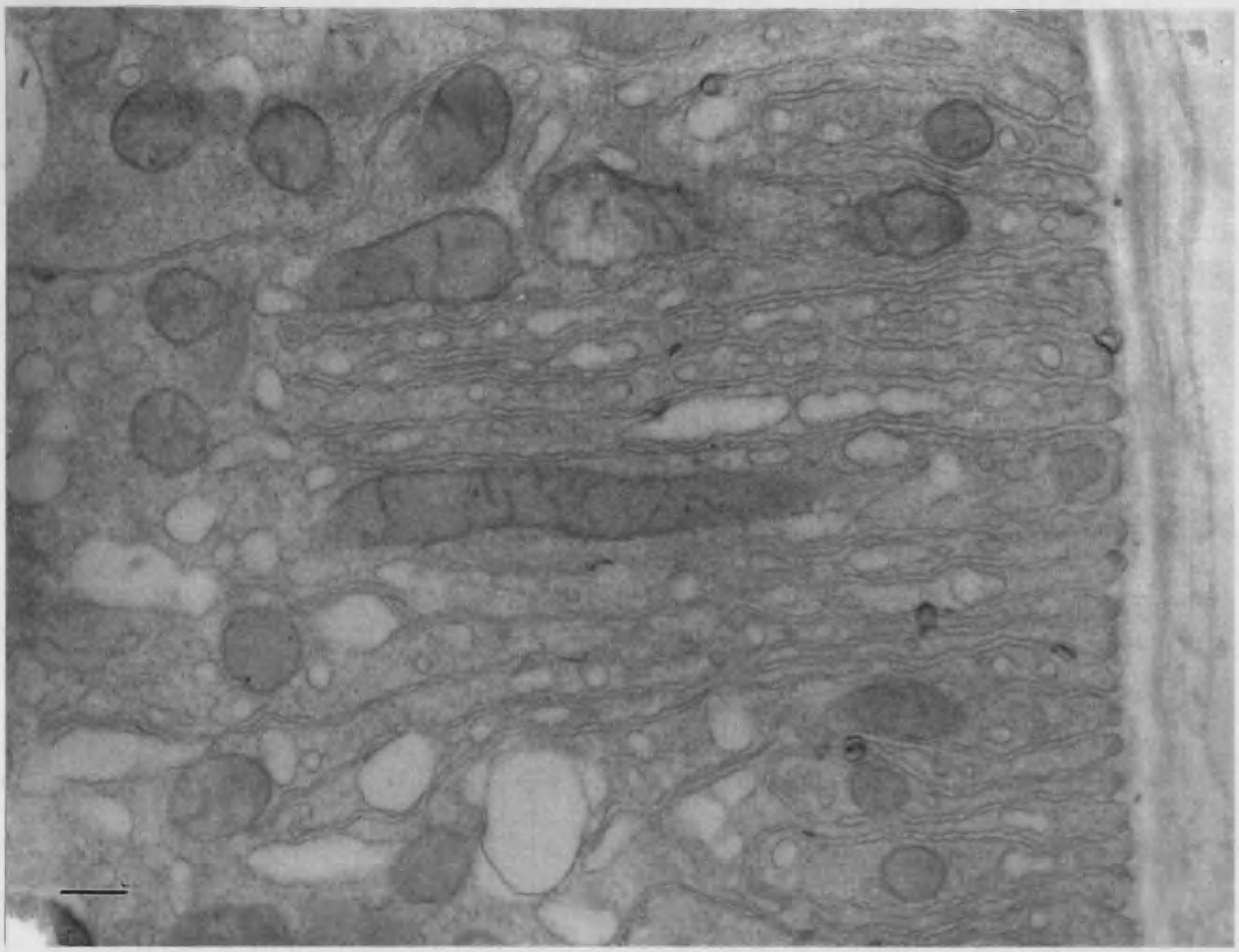

X
X

Figure 6c. The basal membrane infoldings of a control cell (1 h) showing the regular, even nature of the infolds with few vacuoles within them.
Bar = 200 nm.

Figure 6d. The basal membrane infoldings of a control cell (1 h) showing fragmentation and the appearance of many vacuoles (V) within the infolds.
Bar = 200 nm.



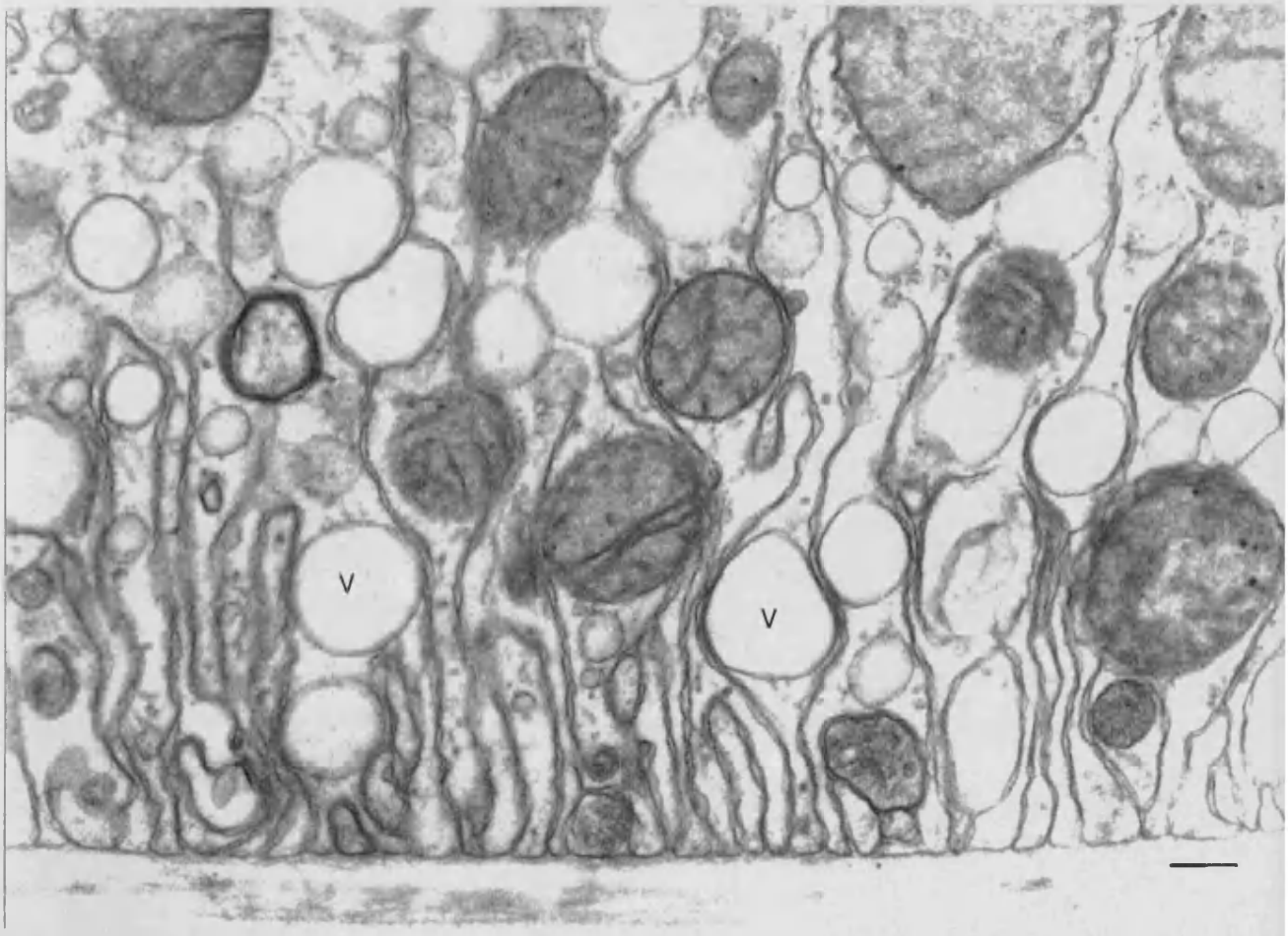
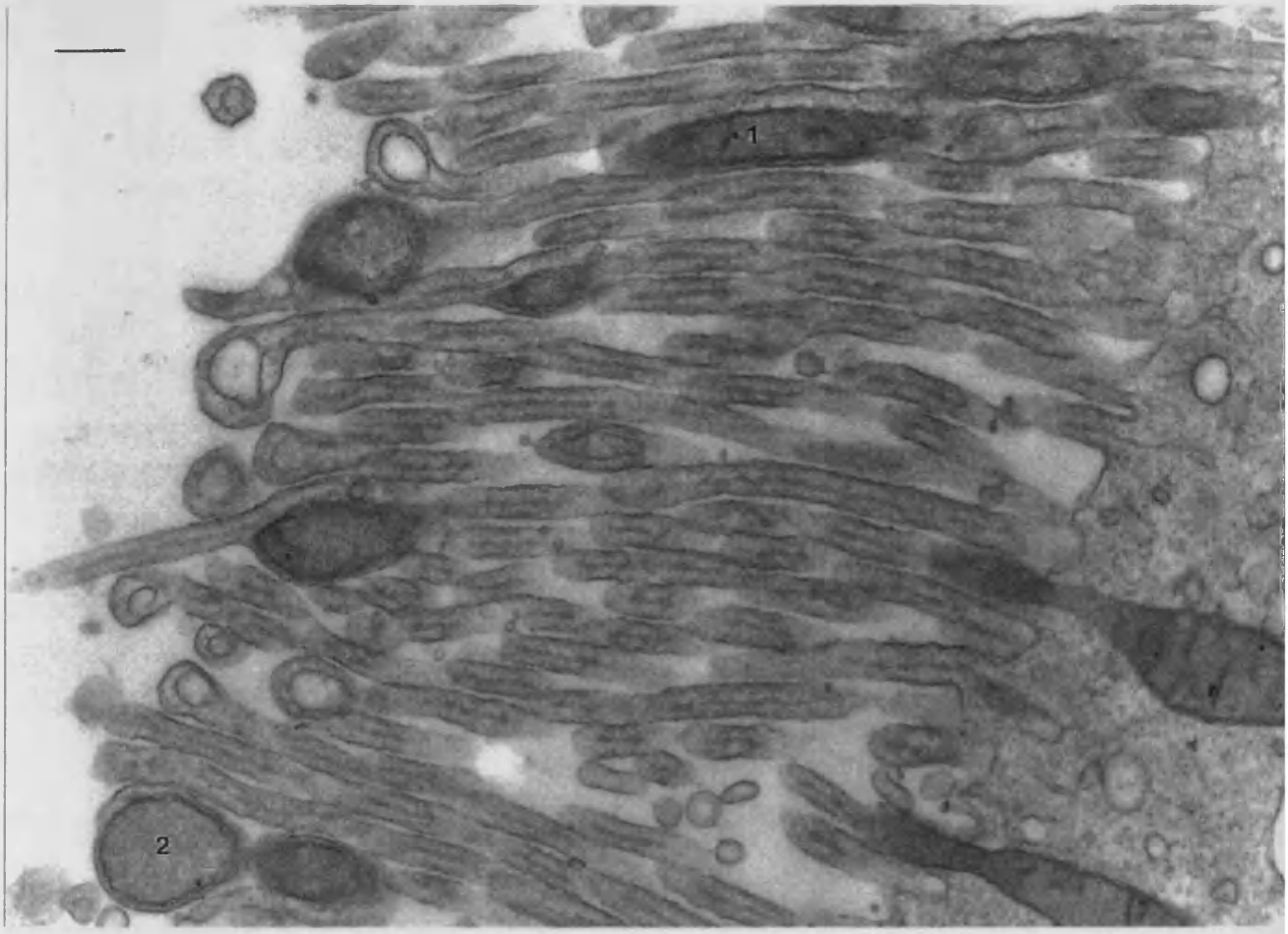
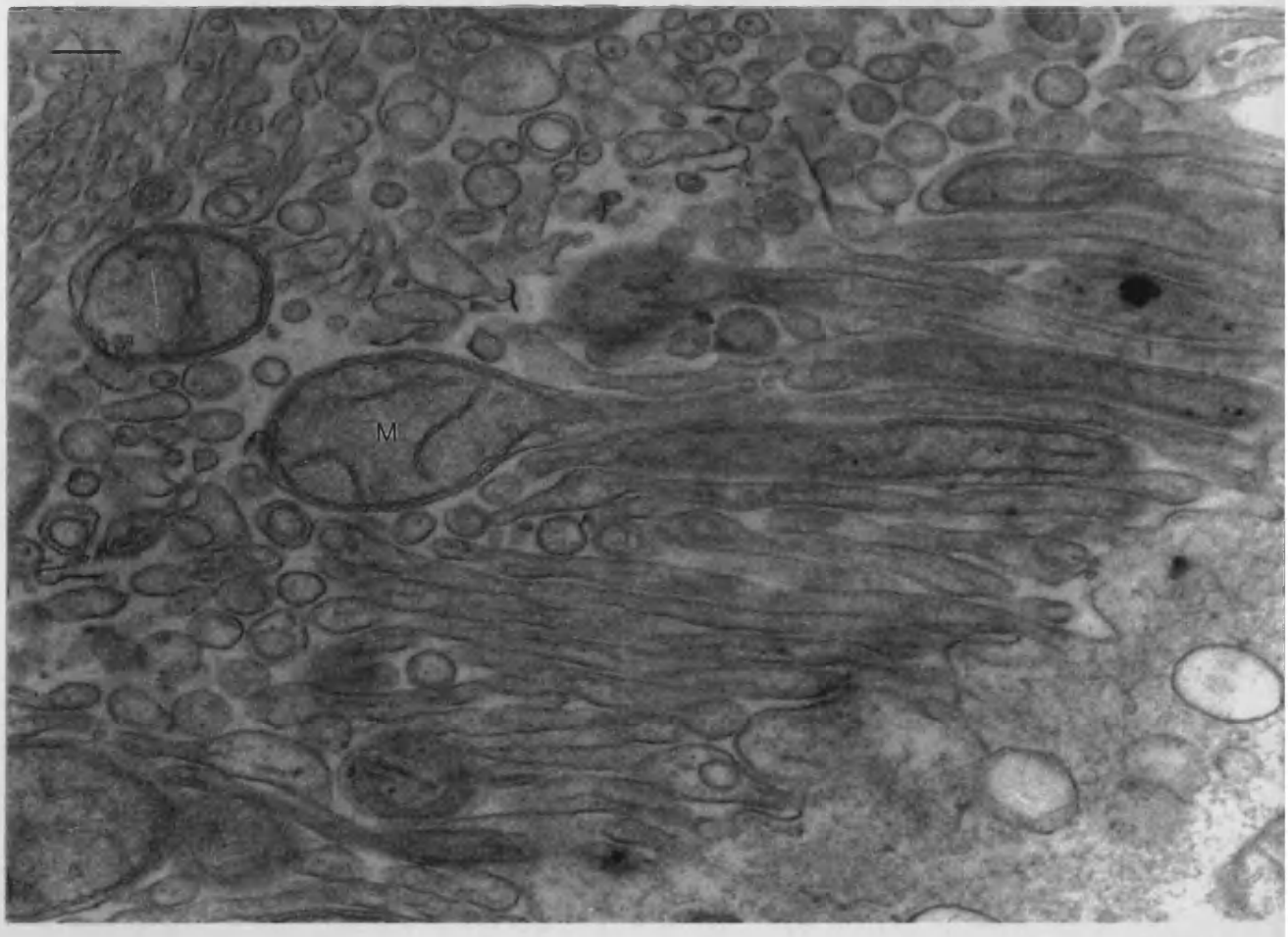

V
V

Figure 7a. The microvilli of a control cell (1 h). The microvilli are regular and even, with thin mitochondria within them (1), but also mitochondria beginning to swell at the tips of some microvilli (2). Bar = 200 nm.

Figure 7b. The microvilli of a destruxin treated tubule cell (1 h) that contain greatly swollen mitochondria (M) within them, and have begun to fragment and fill the lumen. Bar = 200 nm.


1
2

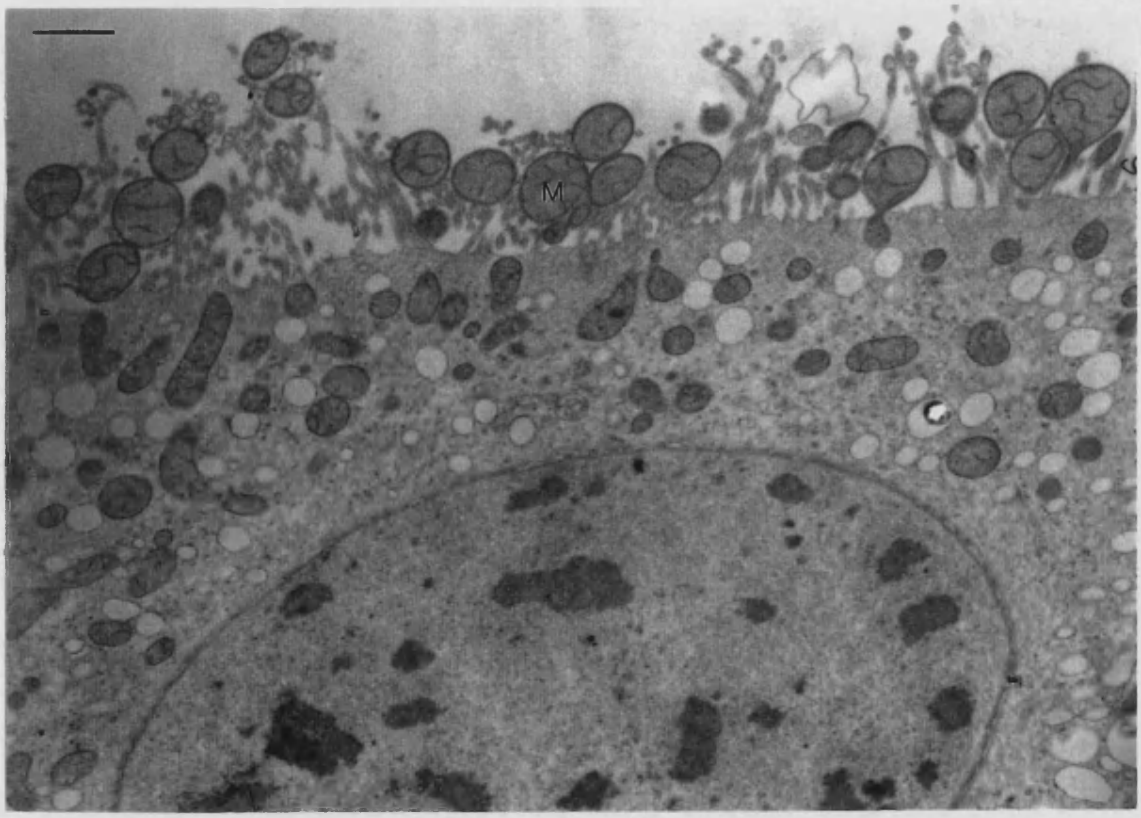

M

the lumen (Fig. 7b), but the majority are fairly regular in appearance, although almost all contain swollen mitochondria. An extreme example of this is shown in Fig. 7c.

After 3 h some of the features noted after 1 h are more pronounced, with little difference between the control and destruxin treated tubules with regards to the cytoplasm and the basal region of the cells. The cytoplasmic mitochondria are heavily swollen with ragged cristae (Fig. 8a and b). The basal membrane infoldings, on most cells, have disintegrated further with greater numbers of vacuoles within the basal region of the cell (Fig. 8c and d).

There are large differences, however, in the appearance of the apical microvilli between the 3 h control and 3 h destruxin treated tubules. Fig. 8e shows the apical region of a typical 3 h control cell, and it can be seen that the mitochondria within the cytoplasm are heavily swollen, but the mitochondria within the microvilli remain unswollen. The microvilli themselves are thin and even. A small number of the 3 h destruxin tubule cells appear as the controls, but the majority have some degree of fragmentation of the microvilli. This ranges from what appears to be the breaking off of swollen mitochondria into the lumen (Fig. 8f), to the complete breakdown of the microvilli, the fragments filling the lumen (Fig. 8g).

Figure 7c. The microvilli of a destruxin treated tubule cell (1 h) that are in tact but contain swollen mitochondria (M) within them. Bar = 1 μm.


M

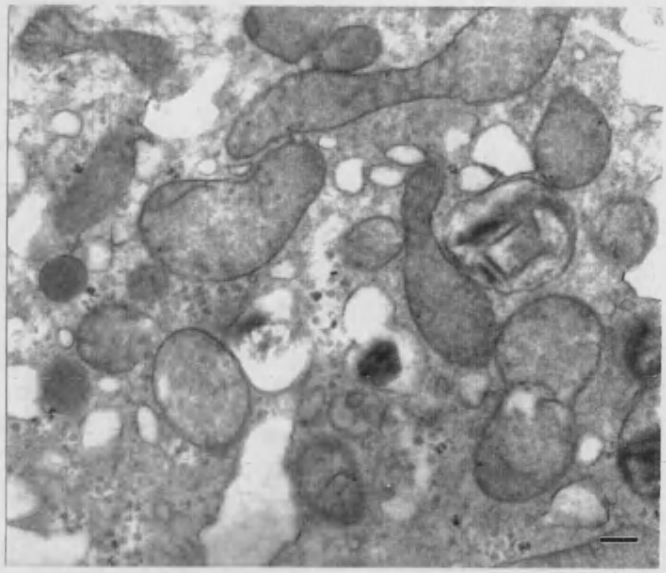


Figure 8a. Cytoplasmic mitochondria of a control cell (3 h). They are heavily swollen with fragmented cristae attached to the outer membrane. Bar = 200 nm.

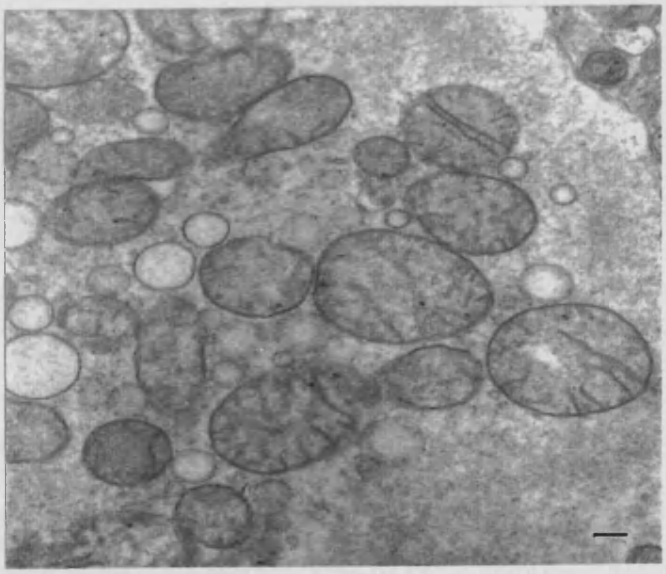
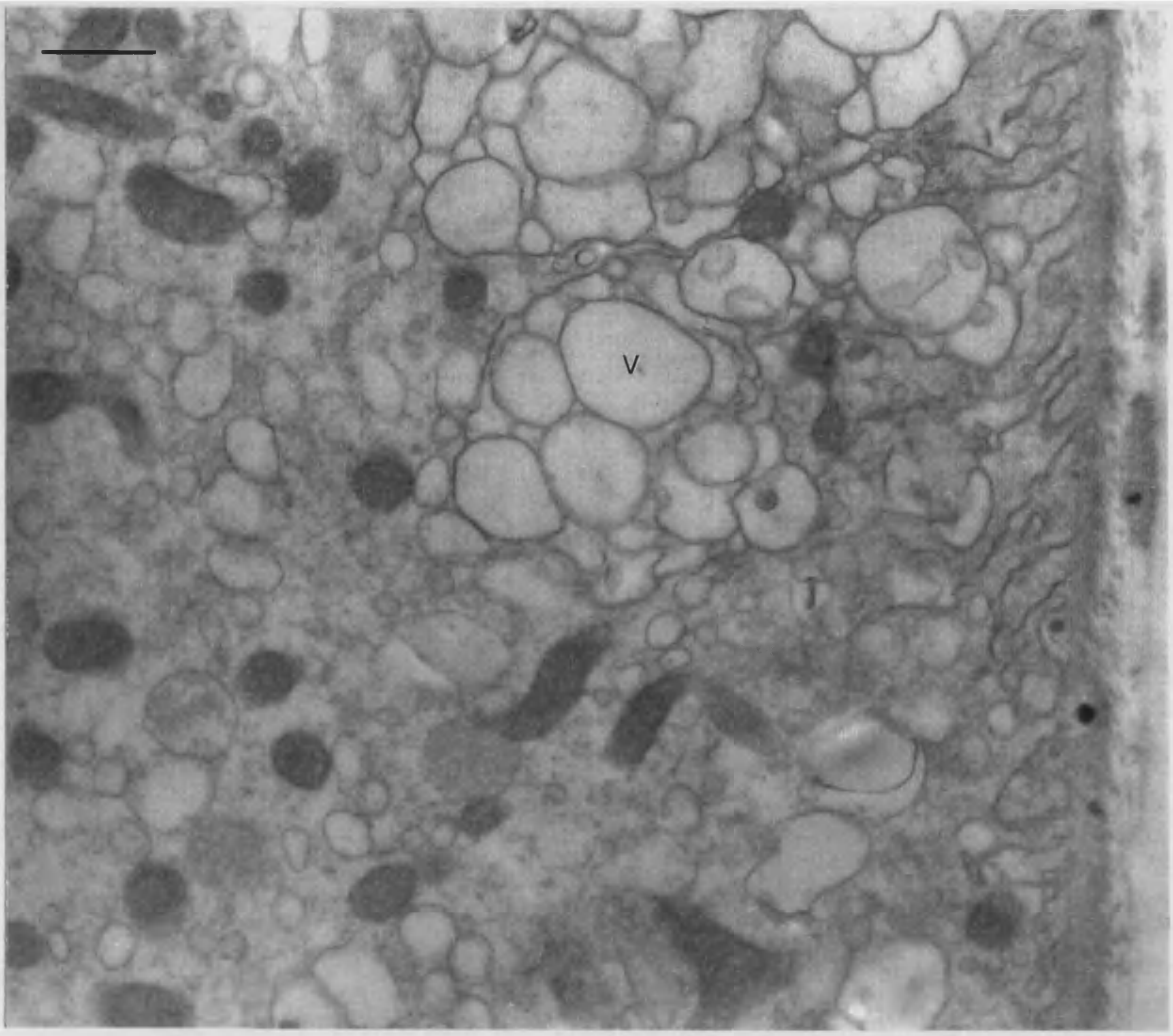


Figure 8b. Cytoplasmic mitochondria of a destruxin treated cell (3 h). They are heavily swollen with fragmented cristae attached to the outer membrane.
Bar = 200 nm.

Figure 8c. The basal membrane infoldings of a control cell (3 h) which have almost completely broken down, with a large number of vacuoles formed (V). Bar = 500 nm.

Figure 8d. The basal membrane infoldings of a destruxin treated cell (3 h) which have almost completely broken down, with a large number of vacuoles formed (V). Bar = 500 nm.


V
T

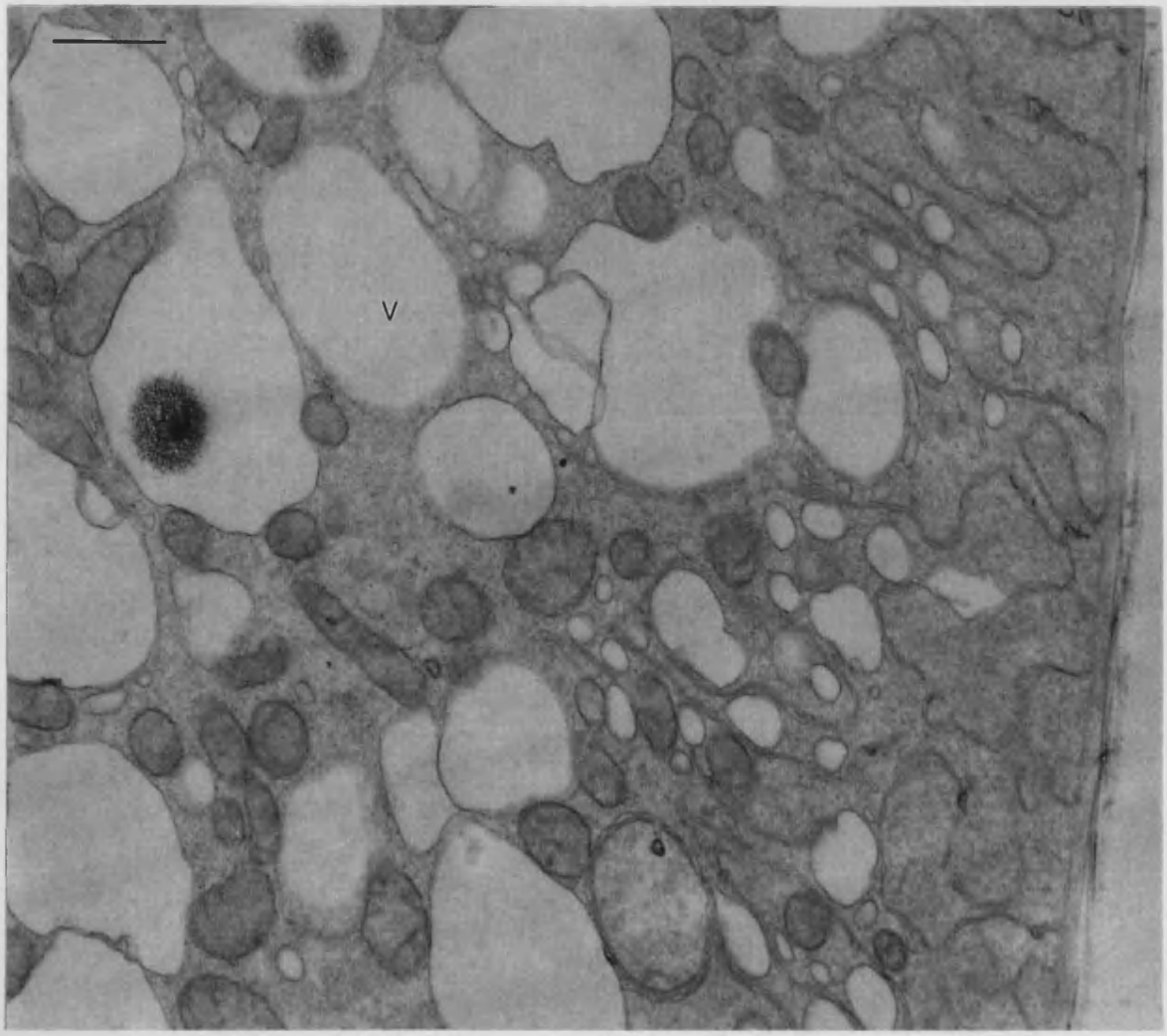
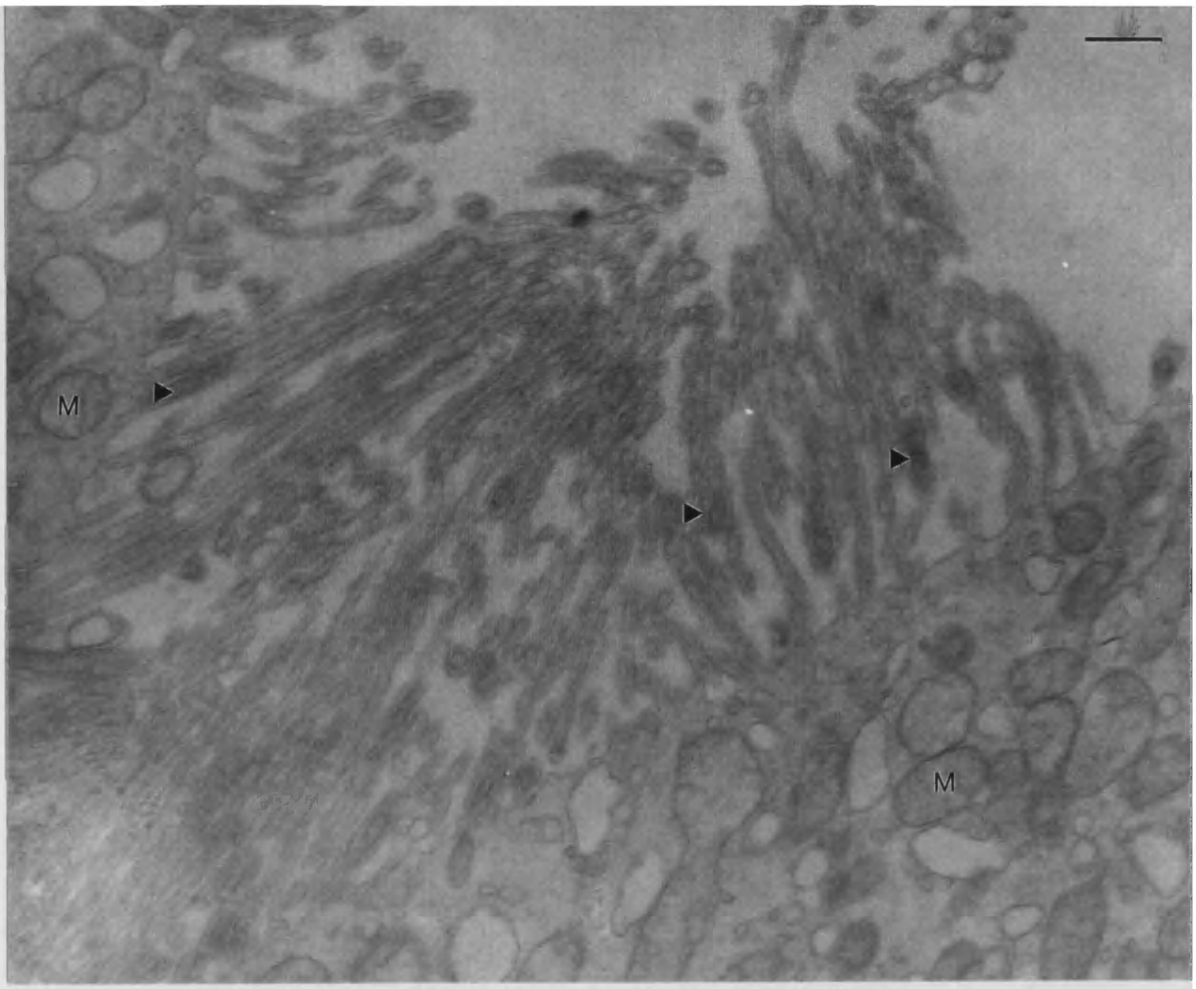

V

Figure 8e. The apical region of a control cell (3 h) showing swollen mitochondria (M) within the cytoplasm and small, regular mitochondria (arrows) within the microvilli. Bar = 500 nm.

Figure 8f. The microvilli of a destruxin treated cell (3 h) show greatly swollen mitochondria (M) which appear to have budded off into the lumen.
Bar = 500 nm.


M
M

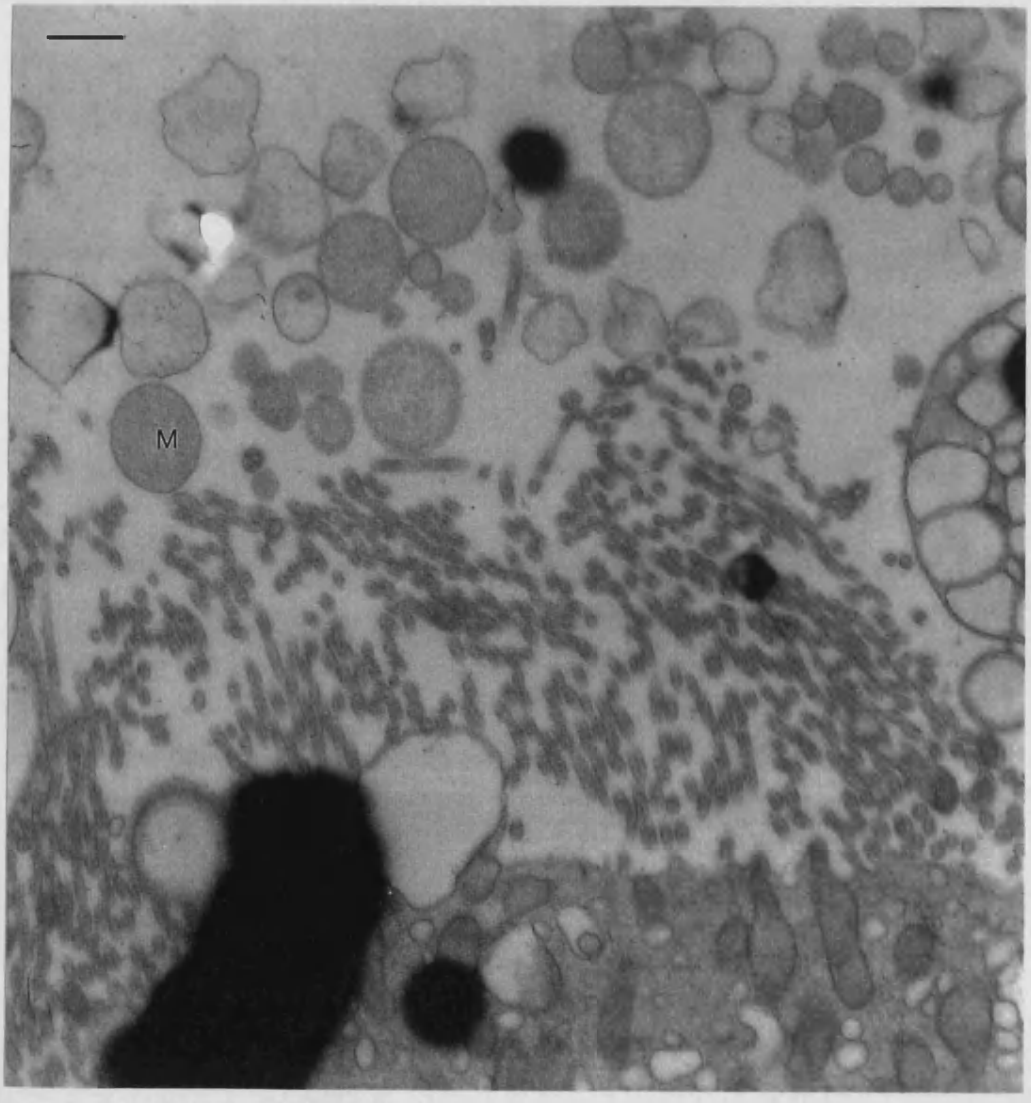
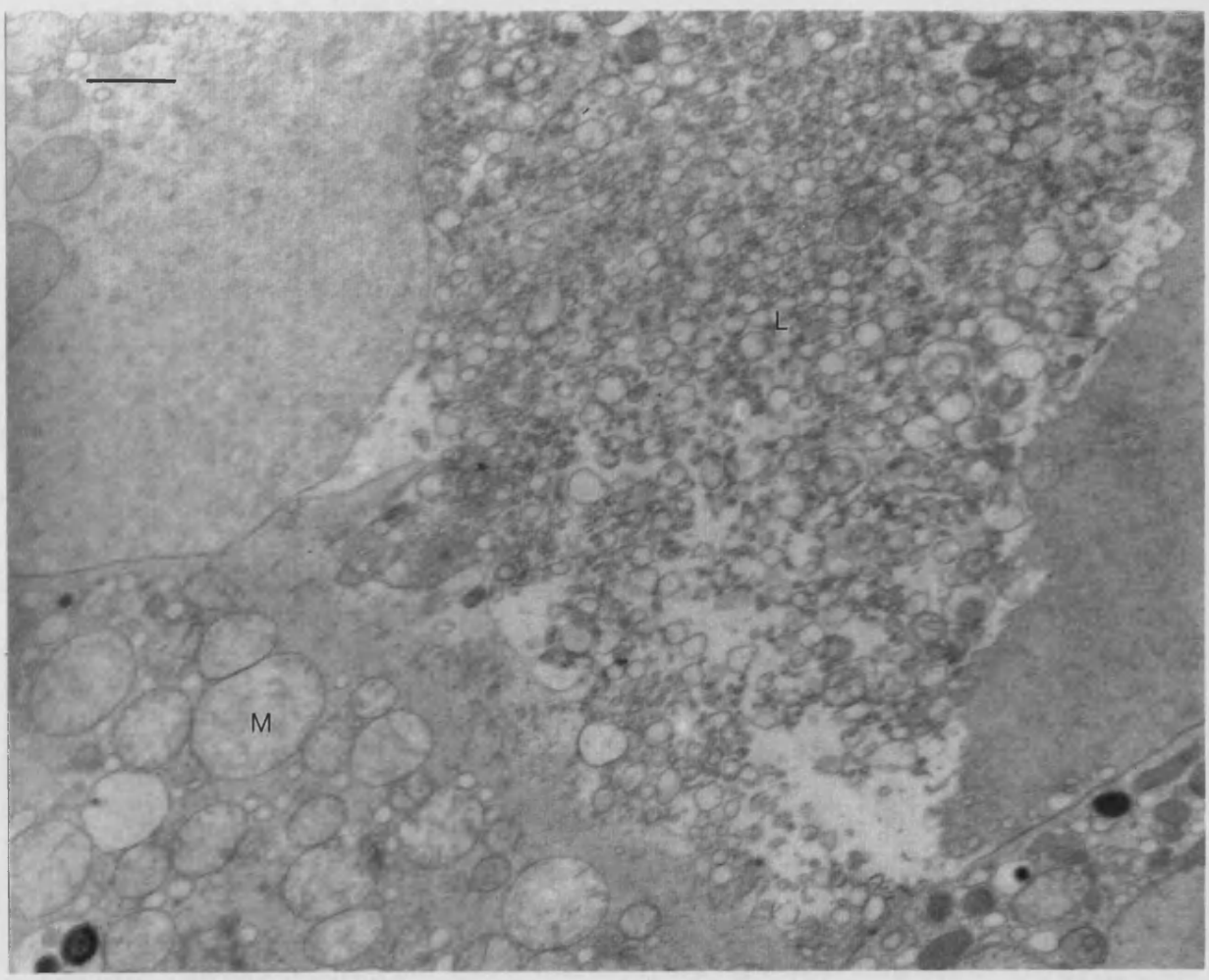

M

Figure 8g. The apical surface of two cells in a destruxin treated tubule (3 h) showing greatly swollen mitochondria (M) within the cytoplasm of the cells and the tubule lumen (L) full of fragmented microvilli and possibly other cell contents. Bar = 1 μm.


L
M

DISCUSSION

The appearance of the 0 min control Malpighian tubules of *S. gregaria* observed in this study is comparable to that in the previously published work of Garrett *et al.* (1988) who examined *S. gregaria*, and also with Martoja (1961) and Charnley (1982) who worked with *L. migratoria* Malpighian tubules. The ultrastructure of the main cell type seen within the *S. gregaria* Malpighian tubules seen here is consistent with the description of the cells previously described by Garrett *et al.* (1988) as primary cells.

The second cell type seen here has some of the features described by Garrett *et al.* (1988) but bears more similarity in overall appearance to the type 2 cell of *L. migratoria* Malpighian tubules described by Martoja (1961) and Charnley (1982). In accordance with both Charnley's (1982) and Garrett *et al.*'s (1988) descriptions, the type 2 cells of the *S. gregaria* tubules seen here are smaller and fewer in number than the type 1 cells, they have basal infoldings that do not penetrate far into the cell, very few, small mitochondria and fewer microvilli with no mitochondria within them. Charnley (1982) also described well developed Golgi and rough endoplasmic reticulum, and a large number of secretory vesicles within the cytoplasm of *L. migratoria* type 2 cells, features seen clearly in the *S. gregaria* type 2 cells here but not described by Garrett *et al.* (1988) in the *S. gregaria* Malpighian tubules they observed. It is possible that these cells are mucocytes and that Garrett *et al.* (1988) saw only the inactive form. Another possibility is that the type 2 cell described here and those of Garrett *et al.* (1988) are different cell types, as Prado *et al.* (1992) believe there are four cell types in locust Malpighian tubules. These are the principal or primary cells (described by Bell and Anstee (1977), Garrett *et al.* (1988) and seen here), stellate cells (described by Garrett *et al.* (1988)), mucocytes (described by Martoja (1961) and Charnley (1982) and seen here) and granulocytes (described by Prado *et al.* (1992)).

The changes that occurred in the ultrastructure of the Malpighian tubule primary cells during the *in vitro* incubation conditions described here are consistent

with the preparation being slightly anoxic. A lack of oxygen would produce an inhibitory effect on ion pumps, interfering with osmoregulation in the cells, so leading to vacuolisation and swelling of the organelles (Trump and Arstila, 1975).

This preparation has been widely used in physiological experiments, but it is the first time the ultrastructure has been studied. The reason this preparation was used was to compare, directly, any ultrastructural changes observed with the physiological effects of destruxin A observed under similar conditions (see chapter 3; James *et al.*, 1993). Actually, in most instances, in the physiological experiments the bathing medium was changed after every 40 min or less, so that ultrastructural changes due to anoxia after 1 h may not be directly comparable. In all the physiological experiments tubules continued to secrete normally although ultrastructural changes due to anoxia may nevertheless have occurred.

There are a number of ways the problem of anoxia can be overcome. In this experiment the presence of the gut in the bathing medium would result in a high rate of oxygen uptake, therefore if individual or groups of isolated tubules were used instead the decrease in oxygen in the bathing medium would be much slower. However, this preparation does have some advantages over isolated tubule preparations. These are minimal handling of the Malpighian tubules and replicate analysis permitted by simultaneous observations taken on a single preparation (Proux *et al.*, 1988; Wheeler and Coast, 1990). In order, therefore, to increase the level of available oxygen in this preparation the bathing medium could be replaced frequently, or oxygen could be bubbled slowly through the bathing medium, as others have done with similar preparations (Reisner *et al.*, 1989; Proux *et al.*, 1988).

Although the effects of anoxia are seen in both the control and treated tubules it appears there are greater differences and changes that occur earlier in the destruxin treated tubules than in the controls. Variability within treatments, a problem encountered by others doing similar EM studies (Reisner *et al.*, 1989) makes this difficult to verify.

Earlier studies on the ultrastructural effects of destruxins have produced results that agree with the slight differences seen here between control and treated tubules after 19 min, 1 h and 3 h. Quiot *et al.* (1985) and Vey and Quiot (1989) stated that in certain cell lines destruxins caused vacuolisation of the cytoplasm and dilated mitochondria with changed cristae. The *in vivo* effects of injected destruxins on the ultrastructure of *C. vomitoria* Malpighian tubules has also been studied previously (S.R. Watkins and A.K. Charnley, unpublished). The destruxins caused the mitochondria to swell and led to the breakdown of the basal membrane infoldings and microvilli, with large vacuoles appearing in the cytoplasm and cell contents being extruded into the lumen. The effects of destruxin A on the structure of the *S. gregaria* Malpighian tubule cells occurring here are somewhat masked by the changes due to anoxia.

The major effect of destruxins observed here is the marked swelling of the mitochondria in the apical microvilli. It must be questioned however, whether these effects are the cause or the consequence of destruxin inhibition of fluid secretion.

The physiological effects of destruxin A apparently precede physical or structural effects. 100 μM destruxin A caused a 70% decrease in the rate of fluid secretion of *S. gregaria* Malpighian tubules *in vitro* in just 5 min, yet there were no changes in the ultrastructure of the tubule cells after 9 min in 100 μM destruxin A and only slight changes after 19 min.

The work of Bradley and Satir (1981) on the Malpighian tubules of *Rhodnius prolixus* has shown that changes in the rate of fluid secretion are paralleled by changes in the structure of Malpighian tubule cells, especially in the position and structure of apical microvilli. In locusts the mitochondria are more evenly distributed within the Malpighian tubule cells making it difficult to ascertain whether any such movement of mitochondria into the apical microvilli occurs.

Perhaps the main conclusion that can be drawn from the present work, and it is an important one, is that the inhibition of fluid secretion seen in *S. gregaria* Malpighian tubules treated with destruxins is not the simple consequence of gross

cellular damage. This suggests that the effects of destruxins are on the regulatory apparatus of the cell, and may be quite specific.

CHAPTER 6. DETECTION OF DESTRUXINS '*IN VIVO*' IN *SCHISTOCERCA GREGARIA.*

INTRODUCTION

Destruxins are the only fungal toxins isolated from mycosed insects in sufficient quantities to cause death (Suzuki *et al.*, 1971). Different *M. anisopliae* isolates are known to produce different amounts of destruxins *in vitro* (Kershaw, 1993) and *in vivo* (Samuels *et al.*, 1988a). Samuels *et al.* (1988a) extracted destruxins from Me1 infected *M. sexta* larvae and found 0.018 μg/ml of presumptive destruxin A.

Although injected destruxins did not have any noticeable effect on *S. gregaria* adults (Samuels *et al.*, 1988a), destruxins have been shown to have physiological effects on *S. gregaria* Malpighian tubules *in vitro* and *in vivo* (see chapters 3 and 4), and also cause ultrastructural changes to the Malpighian tubule cells (see chapter 5). The possible importance of these actions in pathogenesis cannot be gauged until the level of destruxins produced, if they are produced, during *Metarhizium* mycosis of *S. gregaria* is known. In this chapter haemolymph of Me1 mycosed *S. gregaria* is collected and analysed for the presence of destruxins.

MATERIALS AND METHODS

To detect the presence of destruxins in the haemolymph of mycosed locusts the method of Samuels *et al.* (1988a) was used. Adult *S. gregaria* (two weeks after moulting) were infected under the pronotum, with 75,000 spores of *M. anisopliae* isolate Me1 in cotton seed oil. Control locusts were inoculated in the same manner with oil alone. The locusts were then placed in individual containers and kept at 28°C, 30% RH with a 16 h light : 8 h dark cycle for 4 days, with no access to food or water. On day 4 (day 0 being the day of inoculation) the locusts were bled and the blood collected on ice. In an initial experiment, blood samples were pooled to give volumes of 200 to 300 µl each for the infected sample, the positive control and the blank control. To the positive control 100 µl of 100 µg/ml destruxin A in MilliQ water was added. In a second experiment a pool of 1750 µl of infected blood was collected.

The samples were then individually processed by heat fixing at 80°C for 3 min (to deactivate the enzymes and precipitate the blood proteins). After the addition of 1 ml of Ephrussi and Beadle saline, each sample was then thoroughly mixed before being centrifuged at 3300 rpm for 5 min. The supernatant was then loaded onto a C-18 'Sep-pak' cartridge (Waters) (first preconditioned with MilliQ water and acetonitrile) with the aid of a syringe, and eluted with 2 ml 25% and 2 ml 50% acetonitrile successively. The 50% acetonitrile fraction was collected and freeze dried, then stored at -20°C until analysed.

To analyse the fractions they were redissolved in 40% acetonitrile and injected onto an analytical RP-HPLC column. A destruxin A standard was also run.

The presence of destruxins was also assayed using a semi-isolated *Manduca* larval heart preparation (Platt and Reynolds, 1985). Fifth instar *M. sexta* larvae (weighing 8-10 g) were anaesthetised in water for 15 min. The body wall was cut laterally, ventral to the spiracles so that the entire dorsal surface could be removed and pinned out in a sylgard dish. The gut was completely removed and the preparation washed well with several changes of *Manduca* saline.

Movement of the dorsal vessel was monitored by the deflection of a small hook (made from a 1A gauge entomological pin) which was inserted underneath the heart between abdominal segments 5 and 6. This was connected by a light cotton thread to a movement transducer. The transducer's output was recorded on a chart recorder. The preparation was held at an angle of approximately 25^{o} to the horizontal and fresh *Manduca* saline perfused continuously onto the caudal end of the heart at a rate of about 10 ml/min. All test substances were applied directly onto the caudal extremity of the heart. Assays were performed at room temperature (22 ± 2^{o}C).

RESULTS

The HPLC traces are shown in Figs. 1 and 2. The destruxin A standard (2 μg) (Fig. 1a) gave a clear peak with a retention time of 16 min. The spiked control blood (equivalent of 2 μg destruxin A) (Fig. 1b) also showed a clear peak with a retention time of 16 min, although this was slightly smaller than that of the standard, showing that there was a slight loss of destruxin A during the extraction process. Based on peak height, this loss was estimated to be about 15%. Assuming that peaks less than 5-10% of the size of the 2 μg peak in Fig. 1a would not be reliably detected, the lower limit for detection of destruxin A would have been about 150 ng.

In the blank control trace (Fig. 2a) there were no peaks between 15 and 20 min. The first sample (250 μl) of infected blood (Fig. 2b) also contained no noticeable peaks from 15 to 20 min. Assuming that only peaks of 150 ng or more of destruxin A could be detected in infected blood, the Me1 infected locusts must have had a destruxin concentration of less than 0.6 ng/μl in their blood.

This was calculated from only 250 μl of blood from infected locusts. A second batch of locusts were inoculated to provide a greater volume of blood and, perhaps, give detectable levels of destruxin. A volume of 1750 μl of infected blood was collected and processed as before. The RP-HPLC trace is shown in Fig. 3 and again no detectable peaks could be seen within the retention time of 15 to 20 min. This result suggested that if destruxin A was present in Me1 infected blood, its concentration must have been less than 0.085 ng/ml. Nevertheless, further attempts were made to detect the presence of destruxins in HPLC fractions by using the *Manduca* heart bioassay which is very sensitive to the toxins.

The HPLC eluate from the second, larger pool of infected blood was collected to pure 1 minute fractions and freeze dried. The fractions were then each resuspended in 50 μl *Manduca* saline, and 20 μl aliquots assayed on a semi-isolated *Manduca* larval heart bioassay.

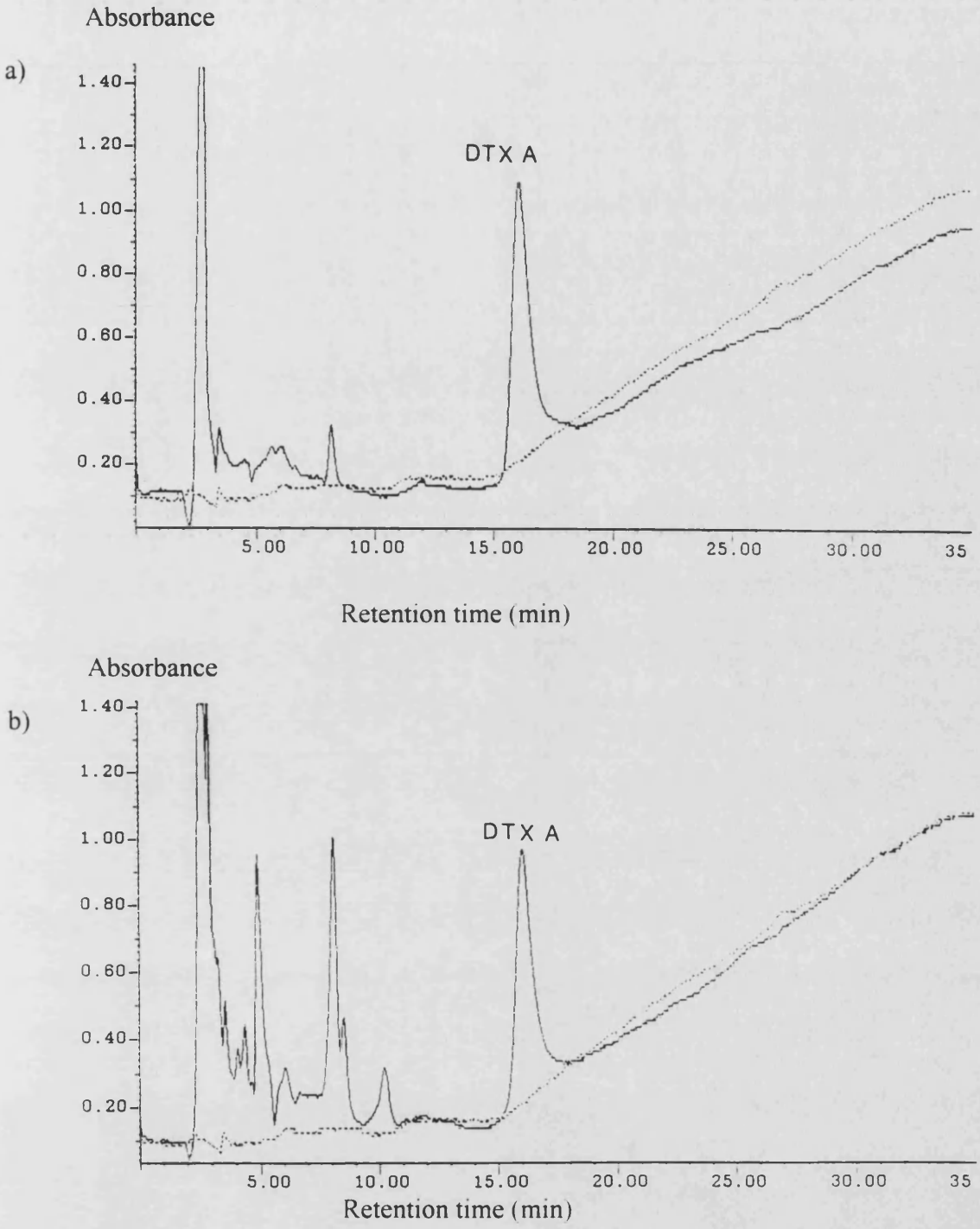


Figure 1. RP-HPLC chromatograms of a) 2 µg destruxin A standard, b) locust haemolymph spiked with 2 µg destruxin A. In both chromatograms the destruxin A (DTX A) can be seen as a clear peak with a retention time of 16 min. The dotted trace on each chromatogram is a blank run.

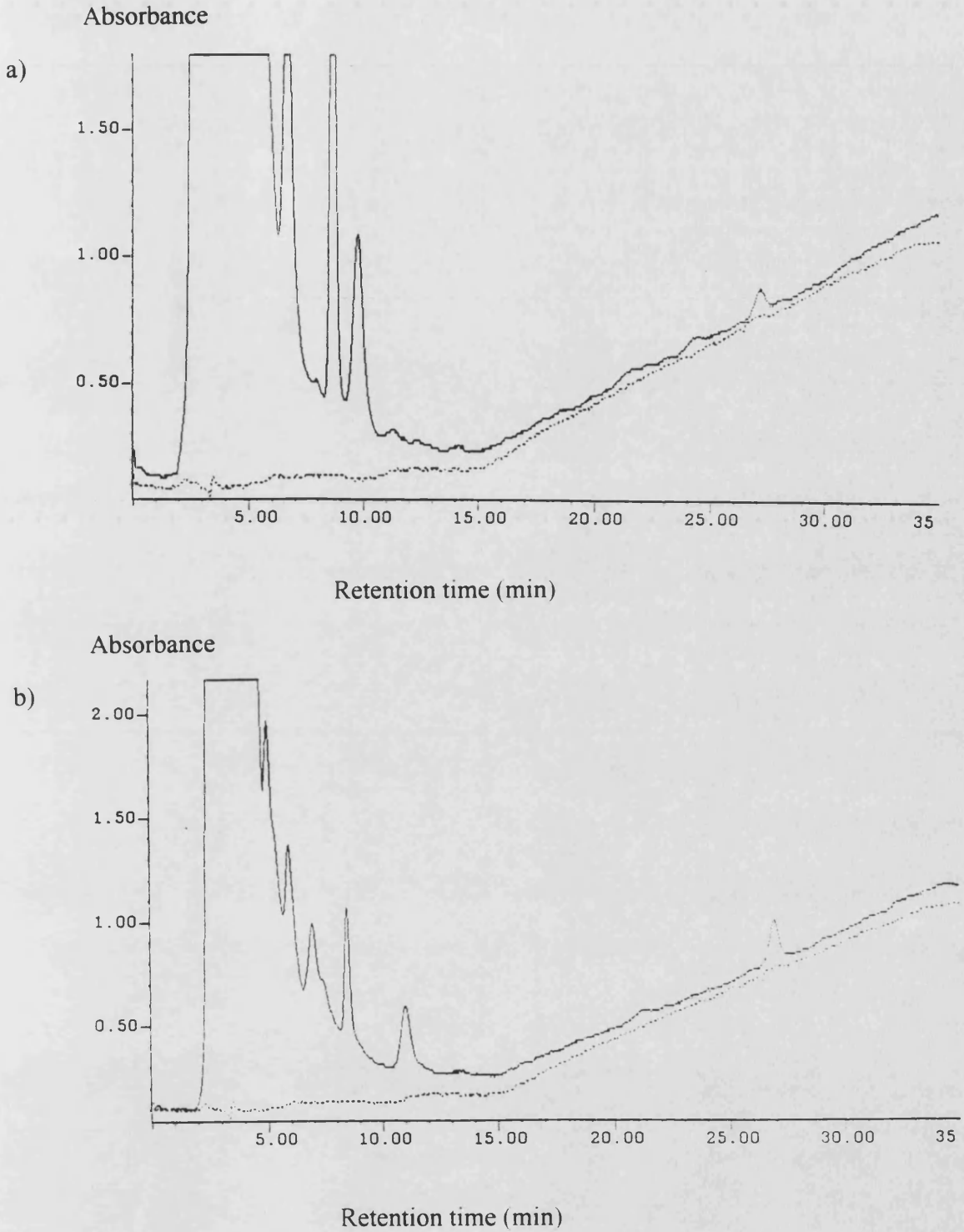


Figure 2. RP-HPLC chromatograms of a) control locust haemolymph and b) haemolymph from Me1 infected locusts. No peaks with a retention time of 16 min can be seen. The dotted trace on each chromatogram is a blank run.

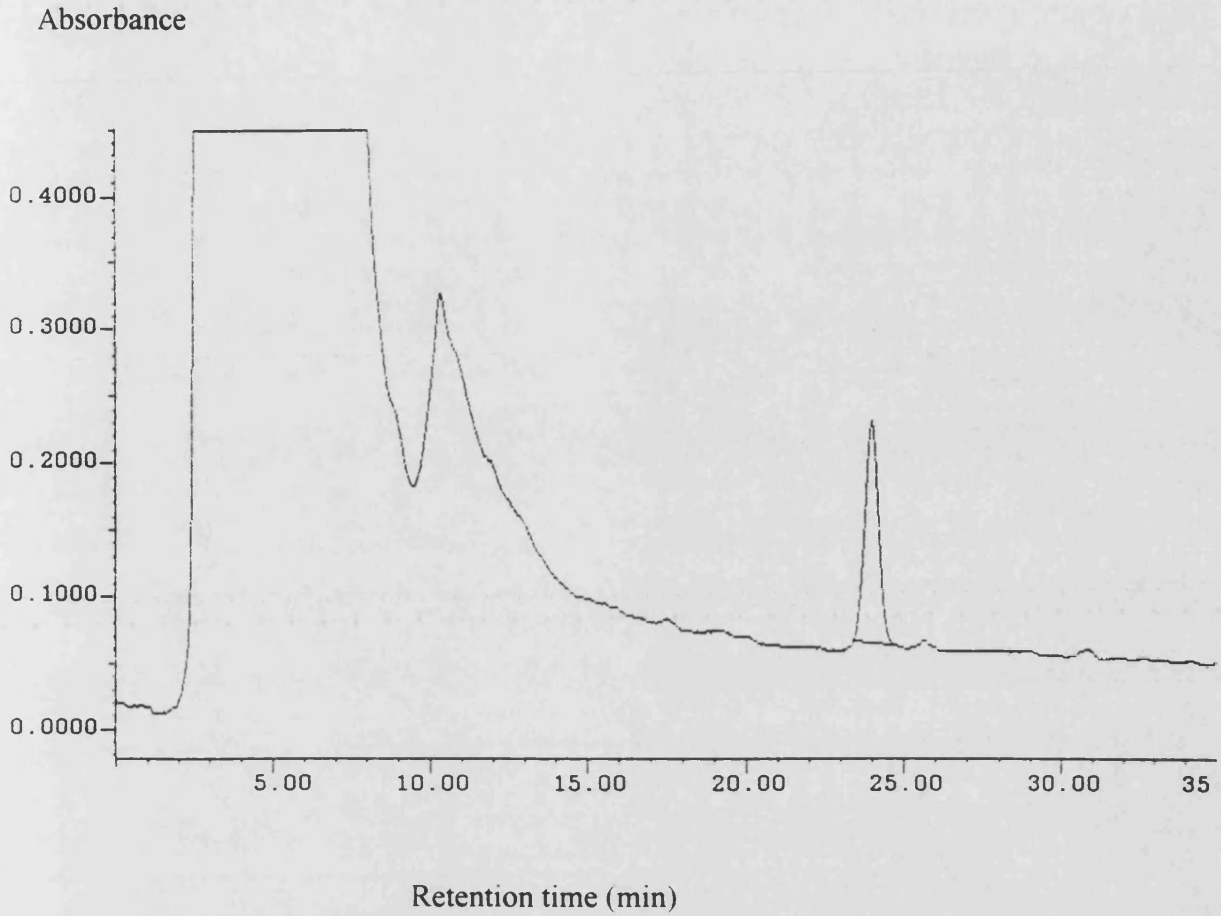


Figure 3. RP-HPLC chromatogram of the equivalent of 1750 µl of Me1 infected locust haemolymph.

Fig. 4 shows the response of the semi-isolated *Manduca* larval heart to a range of doses of pure destruxin A. Destruxin A increased both the rate and the amplitude of the heartbeat in a dose-dependent manner, until with very high doses (40 μg) the heart went into tetanus. By measuring the heart rate for 30s just before and 30s just after the addition of a dose of destruxin A a dose-response curve was constructed (Fig. 5).

The HPLC fractions 1 to 5, from the infected blood, when tested on the larval heart preparation, were seen to accelerate the heart rate. This was probably due to the presence in these fractions of concentrated inorganic ions which pass swiftly through the column. Of the other fractions only numbers 15 and 16 also accelerated the heart rate, each with an increase of 24% (Fig. 6). This response was probably due to the presence of destruxin A. The level of activity seen is similar to that for 4-10 ng destruxin A (see Fig. 4). Each aliquot tested represented 40% of the original fraction. Therefore, it may be estimated that the blood of Me1 infected locusts must have contained 12-28 pg destruxin A/μl. Adjusting for 15% loss on extraction, this figure rises to 14-32 pg/μl.

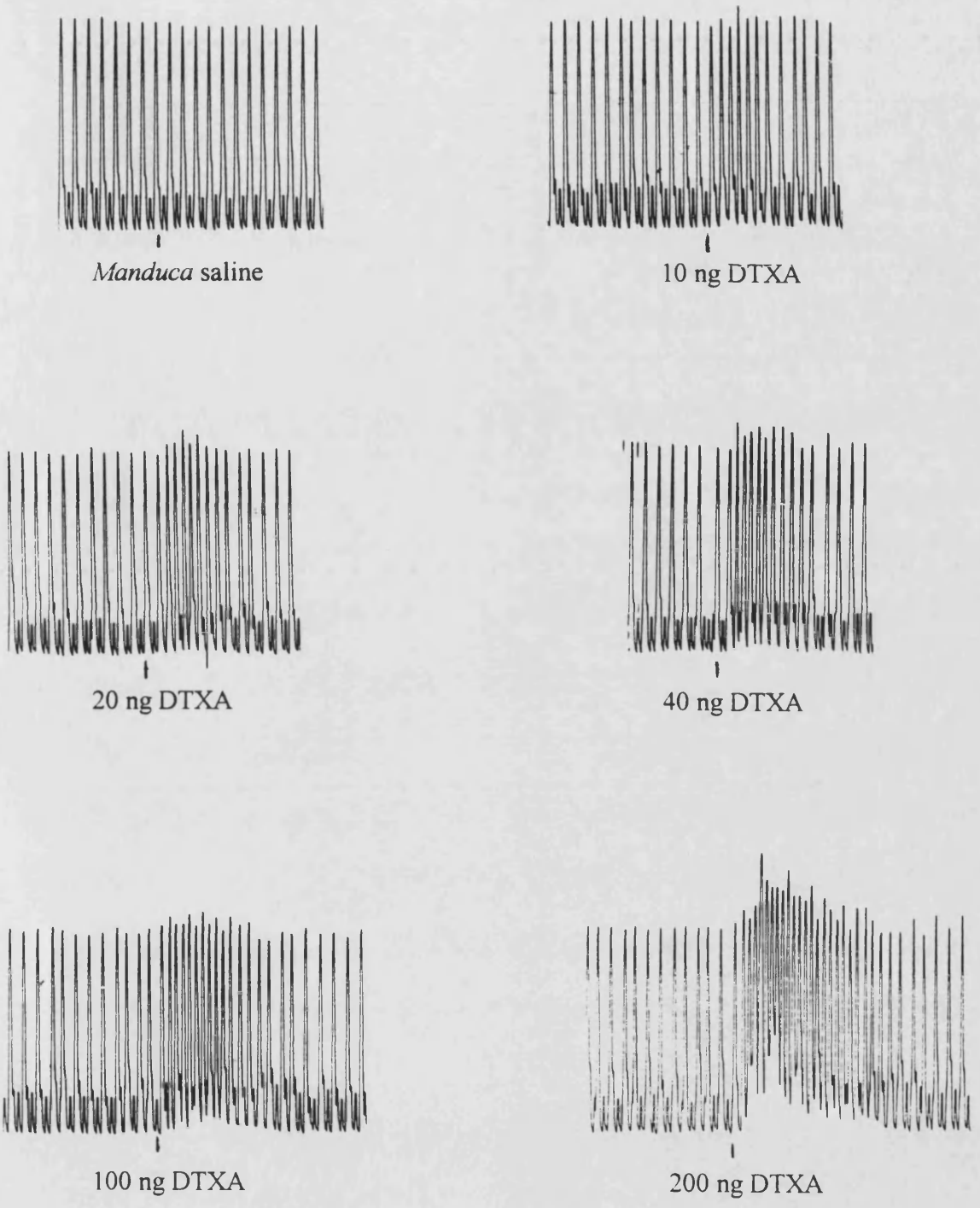


Figure 4. Response of a semi-isolated *Manduca sexta* larval heart preparation to a range of destruxin A (DTXA) doses.

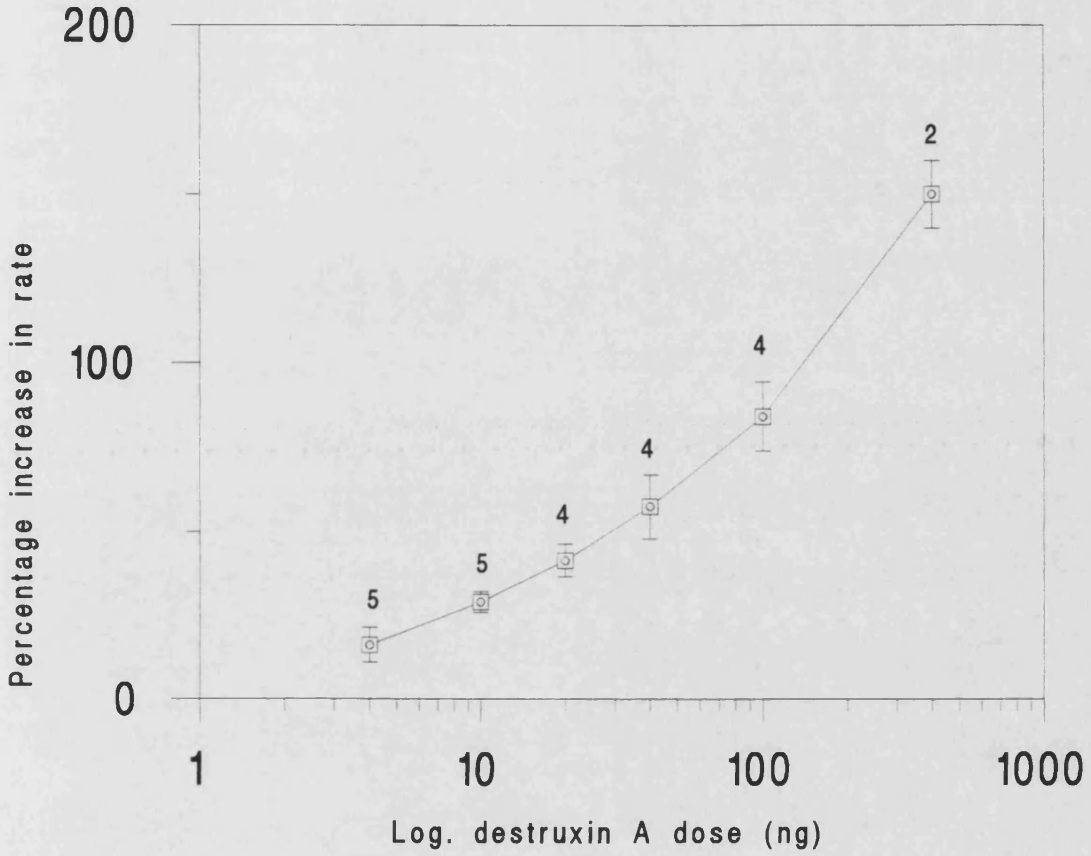
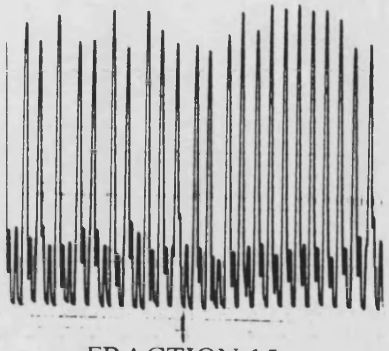


Figure 5. Dose response relationship for the increase in Manduca sexta larval heart rate with applied destruxin A dose. Mean ± SE. Number of replicates for each dose indicated by the numbers on the graph.



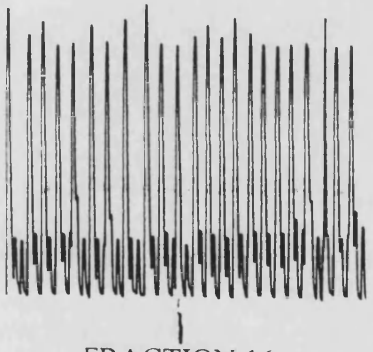


Figure 6. Response of a semi-isolated *Manduca sexta* larval heart preparation to HPLC fractions 15 and 16.

DISCUSSION

Although it was not possible to detect destruxins in the blood of Me1 infected locusts by direct HPLC analysis, using UV light absorption to detect the separated peaks, it was found that the HPLC fractions corresponding to the expected time of elution of destruxin A did indeed contain cardioacceleratory activity like that of the toxin. The amount of toxin found was insufficient to confirm its identity using any physico-chemical method, but its bioactivity and elution time strongly suggest that it was indeed destruxin A. By comparison with a destruxin A dose-response curve, it was estimated that each of 2 fractions contained approximately 4-10 ng of the toxin. This translates to a level of 14-32 pg/µl of presumptive destruxin A in the blood. This is much lower (by 3 orders of magnitude) than the level found in *M. sexta* larvae by Samuels *et al.* (1988a). They found 18 ng/µl of presumptive destruxin A was present in the blood of flaccid paralysed Me1 infected larvae.

A reason for this difference in *in vivo* destruxin levels between locusts and *M. sexta* larvae may be that the locusts have a greater ability to detoxify the destruxin. Cherton *et al.* (1991) and Loutelier *et al.* (1994) showed that locusts were able to detoxify injected destruxins, with noticeable detoxification products present in the tissues only an hour after injection. *M. sexta* larvae are also known to be able to remove injected destruxin from their haemolymph (Samuels *et al.*, 1988a). Fox and Reynolds (1991) showed that the haemolymph of *M. sexta* contains an endopeptidase known to degrade adipokinetic hormone, and this endopeptidase may also be involved in destruxin degradation. In locusts similar enzymes are known to exist (Rayne and O'Shea, 1992), although they are thought to be bound to certain tissues, such as the Malpighian tubules and the ganglia, and degrade passing peptides. Again these enzymes could be involved in destruxin degradation. Without a direct comparison of the fate of injected destruxins in *M. sexta* and *S. gregaria* little more can be said, as both insects appear to have the potential for destruxin degradation.

An obvious difference between the situation in locusts and *M. sexta* is that in the present work the injected insects were not paralysed, while in the experiments of

Samuels *et al.* (1988a) the infected caterpillars were in a state of flaccid paralysis. In part this is because of the lower sensitivity of locusts. Samuels *et al.* (1988a) noted that locusts were much less sensitive to injected destruxins than were *M. sexta* larvae, at least in this respect. But evidently there is also much less toxin present.

The estimated concentration of 14-32 pg/µl destruxin A in the blood of the infected locusts would be insufficient to inhibit Malpighian tubule function. The experiments described in chapter 3 found that the IC_{50} for destruxin A inhibition of fluid secretion by locust Malpighian tubules *in vitro* was ca. 23 µM (or 11.5 ng/µl), far higher than the levels found here.

The picture of destruxin production and detoxification *in vivo* is a complicated one, and further investigation of both of these processes will help in understanding the role of destruxin in pathogenesis in different insect species.

CHAPTER 7. GENERAL DISCUSSION.

The search for virulence determinants should be an integral part of the development of mycoinsecticides, since this information will aid the selection of optimum strains and, in the future, the development of more aggressive, faster killing fungal pathogens. However, unravelling the basis of isolate virulence is complicated, particularly for single gene products, because of the diverse genetic background of fungi like *Metarhizium* spp. (Cobb and Clarkson, 1993). In the present work, for the first time, a detailed study has been made of the prepenetration and penetration stages of infection of a number of *Metarhizium* spp. in a semi-*in vivo* model system that allows good replication of experiments carried out under carefully controlled conditions. Despite the attention to detail in the design and execution of this quantitative study the only significant correlation was that found between the rate of germination and isolate virulence.

The time of initial appressorium formation varied greatly between isolates, though the time span over which initial penetration occurred was much less. *A priori* it might be thought that the latter stage should be more important than the former, but neither correlated significantly with virulence. The number of penetrant hyphae per infective propagule may be critical to penetration, but again there was no significant correlation with isolate virulence.

The production of infection structures involves many genes. In contrast fungal proteases are single gene products. Therefore it is less surprising that no significant correlation was found between protease production and isolate virulence. However, in these experiments enzyme was extracted from infected wings at the time at which hyphae had already successfully penetrated the cuticle. It may be that this was too late. A more extensive study of the levels of proteases produced during different stages of infection, possibly linking these levels with numbers of appressoria and penetration pegs, might shed more light on the involvement of proteases in virulence.

An alternative approach is to observe the impact of specific inhibitors of the proteases *in situ* during mycosis on isolate virulence. Overall it would seem from the present work that I must conclude with Jackson *et al.* (1985) and Charnley and St. Leger (1991) that virulence is not determined by a single factor but is the result of a combination of factors that act additively.

The way forward from here certainly seems to be with molecular biology, perhaps using gene disruption to knock out the production of a particular cuticle-degrading enzyme or enzyme complexes and observe the effects on virulence.

The actual ability of a fungus to penetrate the cuticle may be a key step in pathogenesis, but factors involved later in mycosis, e.g. within the haemocoel, may have a greater bearing on virulence.

Destruxins may also be involved in the virulence of *Metarhizium* spp. for the desert locust. A compound thought to be destruxin A was found in mycosed adult locusts, although at levels far below those found in mycosed *M. sexta* larvae (Samuels *et al.*, 1988a). This may be due to the locust having a greater ability to detoxify the destruxins, but this is an area that requires further investigation. A comparative study of the detoxification of destruxins in locusts and *M. sexta* would help elucidate the differences between the role of destruxins in mycosis of the two insect species. This could involve observing the fate of injected destruxins using HPLC techniques and/or radiolabelled toxin.

Locust muscle is much less susceptible to destruxins than Lepidopteran muscle (Samuels *et al.*, 1988a). Given the low level of destruxins in mycosed locusts it would seem that destruxins cannot act on muscles during pathogenesis. This is confirmed by observations of infected locusts which did not, on the whole, reveal symptoms of paralysis. Although locust Malpighian tubule function was inhibited by destruxins, both *in vivo* and *in vitro* in this study, the level required was much higher

than that found in mycosed locusts. This suggests that destruxins have little effect on these tissues also during mycosis.

Destruxins caused minor ultrastructural alterations in the cells of locust Malpighian tubules *in vitro*, but again the concentrations involved were higher than those in mycosed locusts, suggesting that these effects of destruxins have little bearing on pathogenesis in locusts.

The levels of destruxin in *M. sexta* and *S. gregaria* are very different. Destruxins are thought to play a direct role in the pathogenesis of *M. sexta* larvae (Samuels *et al.*, 1988a). Kershaw (1993) observed a relationship between isolate virulence towards *S. gregaria* and destruxin production, suggesting that despite the low titre destruxins may be involved in *Metarhizium* mycosis, perhaps by interfering with the immune system (Huxham, *et al.*, 1989), although destruxins were tested by Huxham *et al.* (1989) at levels higher than those found in mycosed locusts. It is possible that localised high concentrations of destruxins in tissues in which large amounts of fungal hyphae/blastospores are present may lead to effects at specific sites, including the Malpighian tubules, and therefore aid pathogenesis. It would appear, however, that at present, there is little evidence for the involvement of destruxins in the pathogenesis of *S. gregaria*, other than on the immune system. This is an area that could be investigated further.

In the present study destruxins were seen to act *in vitro* at a level beyond the control of calcium and cAMP in locust Malpighian tubules. This action appears to be different from that against *M. sexta* muscle (Samuels *et al.*, 1988b), though even here the involvement of the ion channels may be an indirect expression of destruxin action. The effects of destruxins on the structure of locust Malpighian tubule cells implies the possible action of destruxins on the regulation of cellular function and it would be of interest to pursue this further.

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APPENDIX I

Locust rearing conditions

Temperature : room = 28°C

cage = 32 - 36°C

Light/dark cycle = 16 h day

Diet : bran - 10:1 with dried yeast, sprayed with a solution of sodium sulfamethazine (4.26% w/v), sodium sulfathiazole (3.65% w/v) and sodium sulfamerazine (3.13% w/v) for control of *Malamoeba locustae* (Henry and Oma, 1968).

wheat - fresh

water - with 5% solution of the same triple sulphur solution added to bran.

Eggs : incubated at 34°C

Locust saline

(from Maddrell and Klunsuwan, 1973)

	g/l
$CaCl_2$	0.30
NaCl	5.73
KCl	1.50
$MgCl_2.6H_2O$	0.41
Glucose	1.80
$NaHCO_3$	1.86
$NaH_2PO_4.2H_2O$	1.09
Na glutamate	0.83
Na citrate	0.88
Malic acid	0.37

Dissolve all but $CaCl_2$ in approx. 900 ml distilled water, adjust pH to 7.2, add $CaCl_2$ and make up to 1 l with distilled water. Filter sterilise.

Sabouraud's Dextrose Agar (SDA)

	full strength	1/4 strength
Dextrose	40 g	10 g
Mycological peptone	10 g	2.5 g
Yeast extract	5 g	5 g
Agar (No.3)	20 g	20 g
Distilled water	1 l	1 l

Mix and boil until dissolved, adjust pH to 6.5, sterilise at 15 psi for 15 min.

Water agar

Agar	20 g
Distilled water	1 l

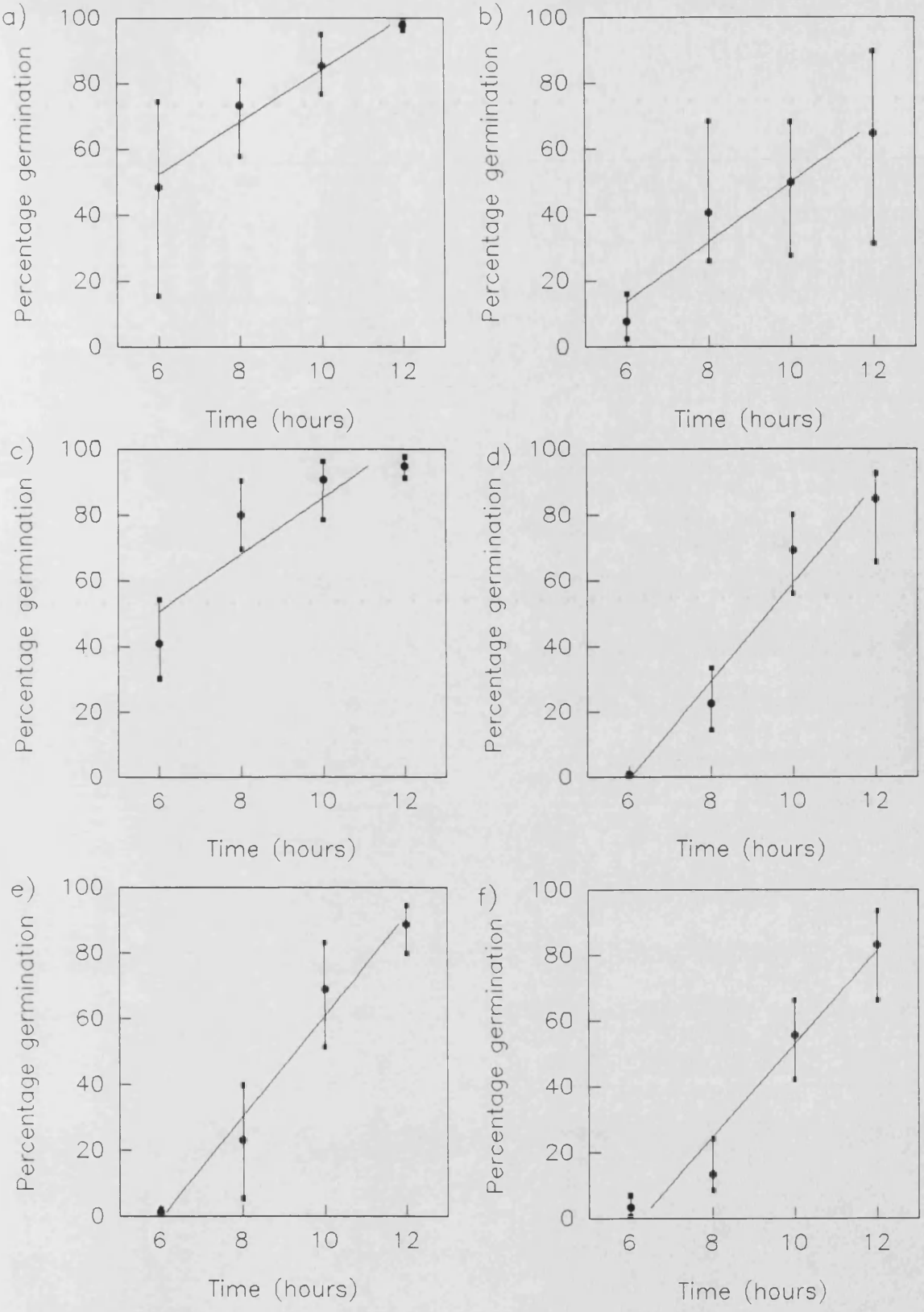
Make as for SDA.

APPENDIX II

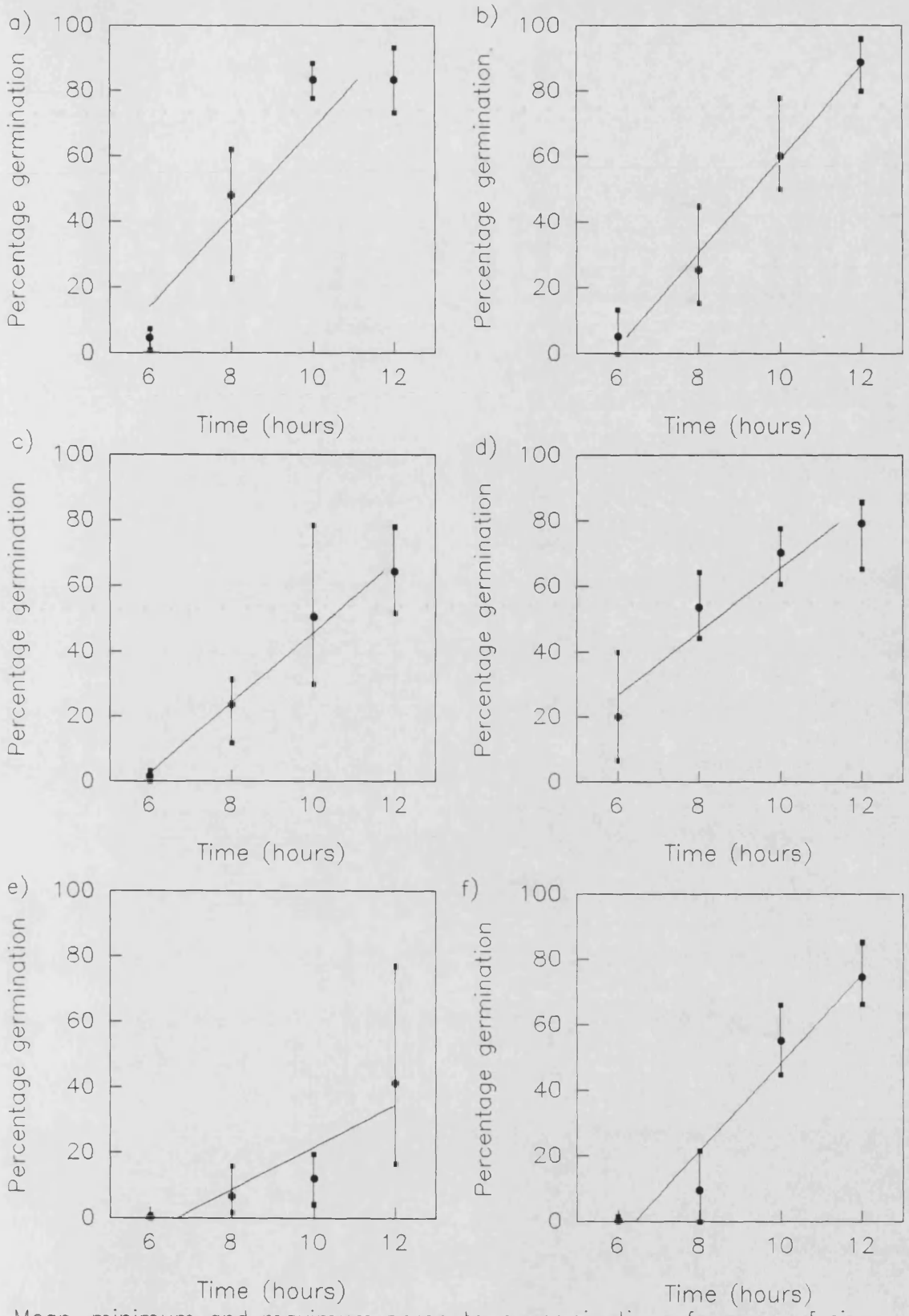
Graphs of mean, maximum and minimum percentage germination from 6 to 12 h after inoculation for all nineteen *Metarhizium anisopliae* and *flavoviride* isolates on excised locust wings.

Graphs of the number of appressoria per spore produced during initial appressoria formation for sixteen *Metarhizium anisopliae* isolates and two *M. flavoviride* isolates on excised locust wings.

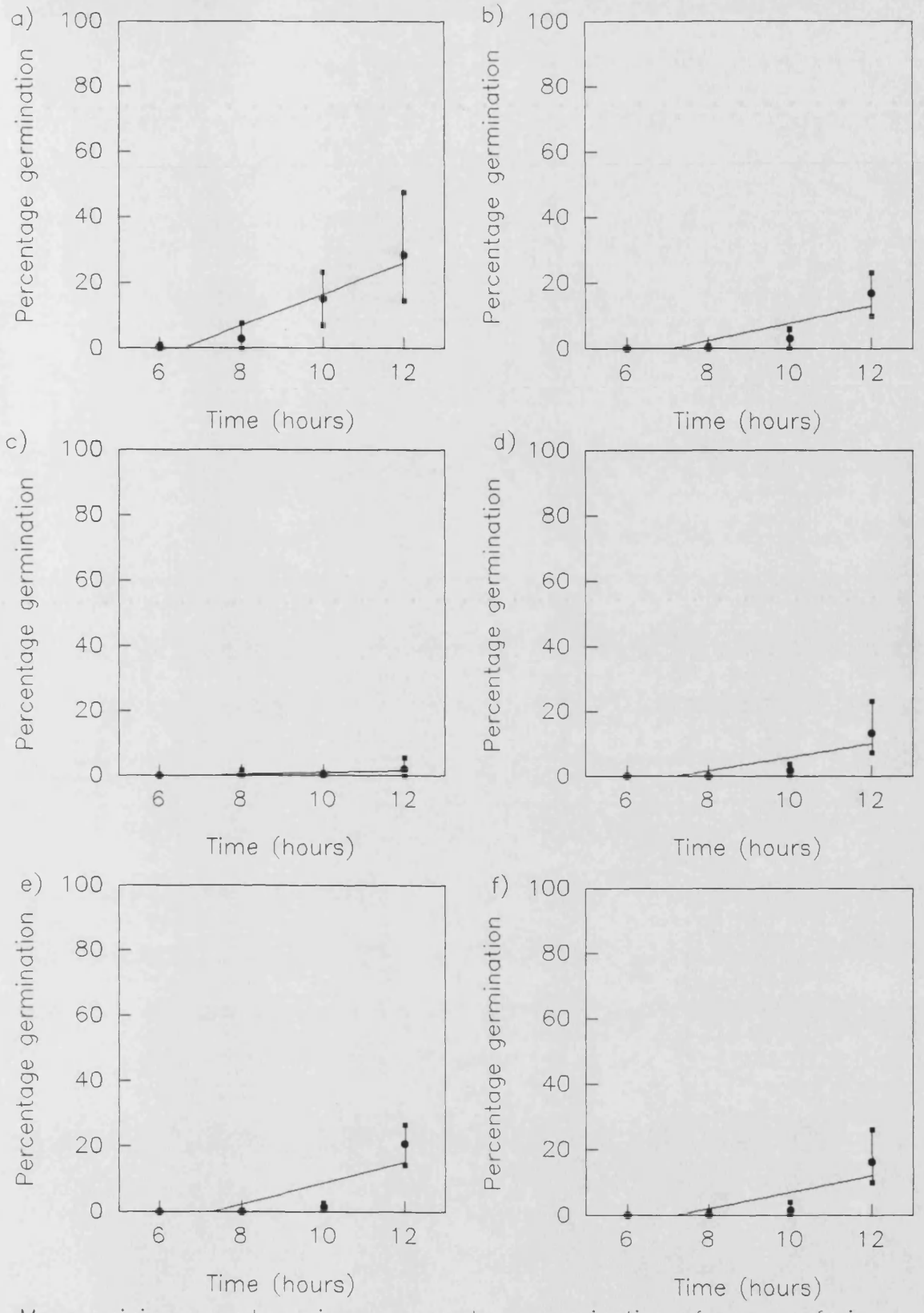
Graphs of the number of penetration points per spore produced during initial penetration for sixteen *Metarhizium anisopliae* isolates on excised locust wings.



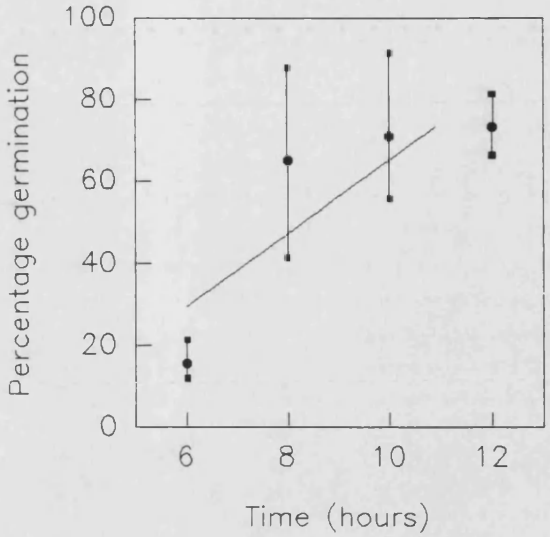
Mean, minimum and maximum percentage germination of spores of six Metarhizium isolates, 6, 8, 10, and 12 hours post inoculation, incubated at 27°C on excised locust wings. M. anisopliae isolates a) Me1, b) 168777ii c) 298061 and M. flavoviride isolates d) Arsef 324, e) 330189 and f) 324673. Regression lines have been fitted to the data.



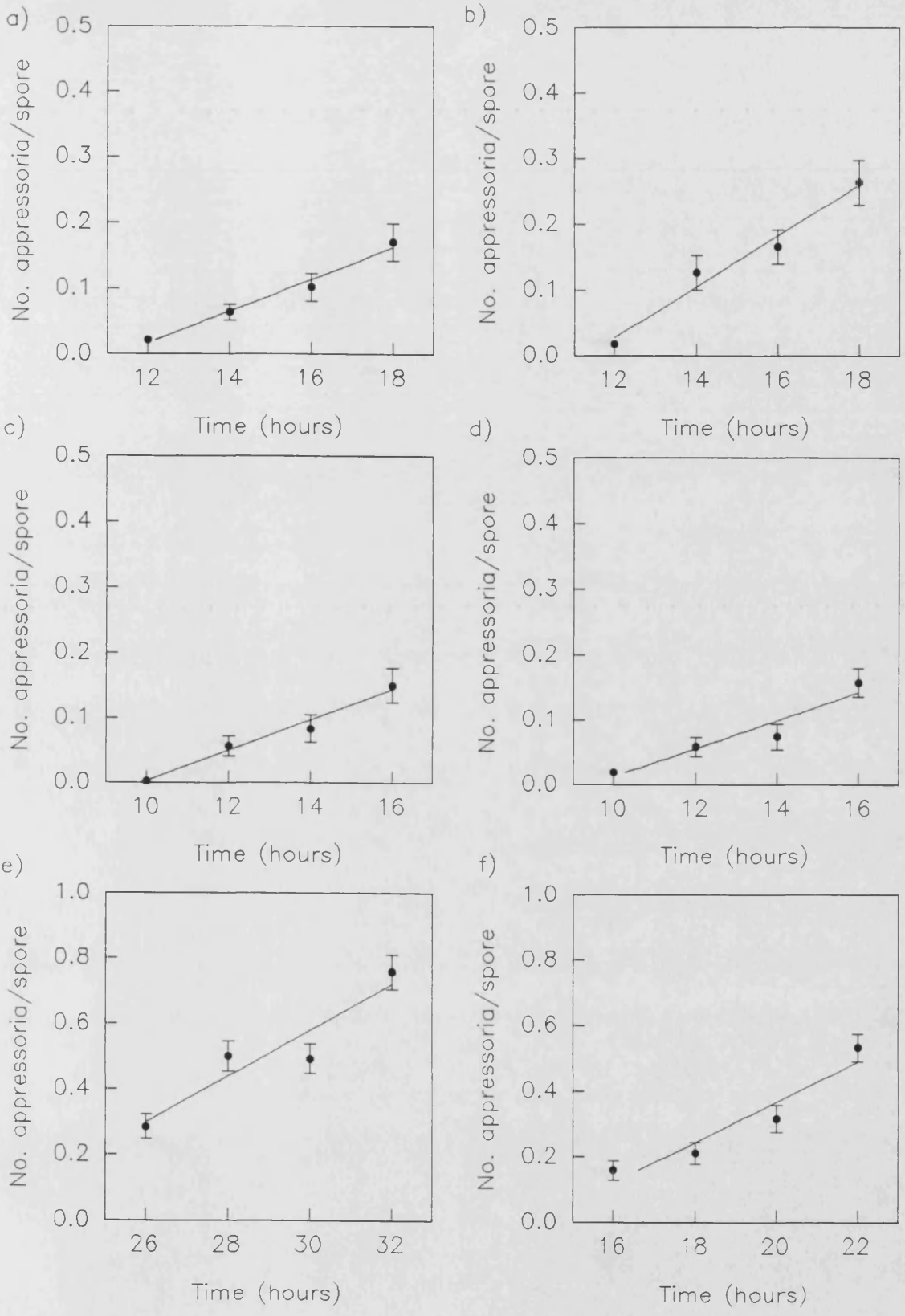
Mean, minimum and maximum percentage germination of spores of six Metarhizium isolates, 6, 8, 10, and 12 hours post inoculation, incubated at 27°C on excised locust wings. M. anisopliae isolates a) 299984, b) 299981, c) Arsef 727, d) I91 676, e) Nr 48 and M. flavoviride isolate f) Arsef 2023. Regression lines have been fitted to the data.



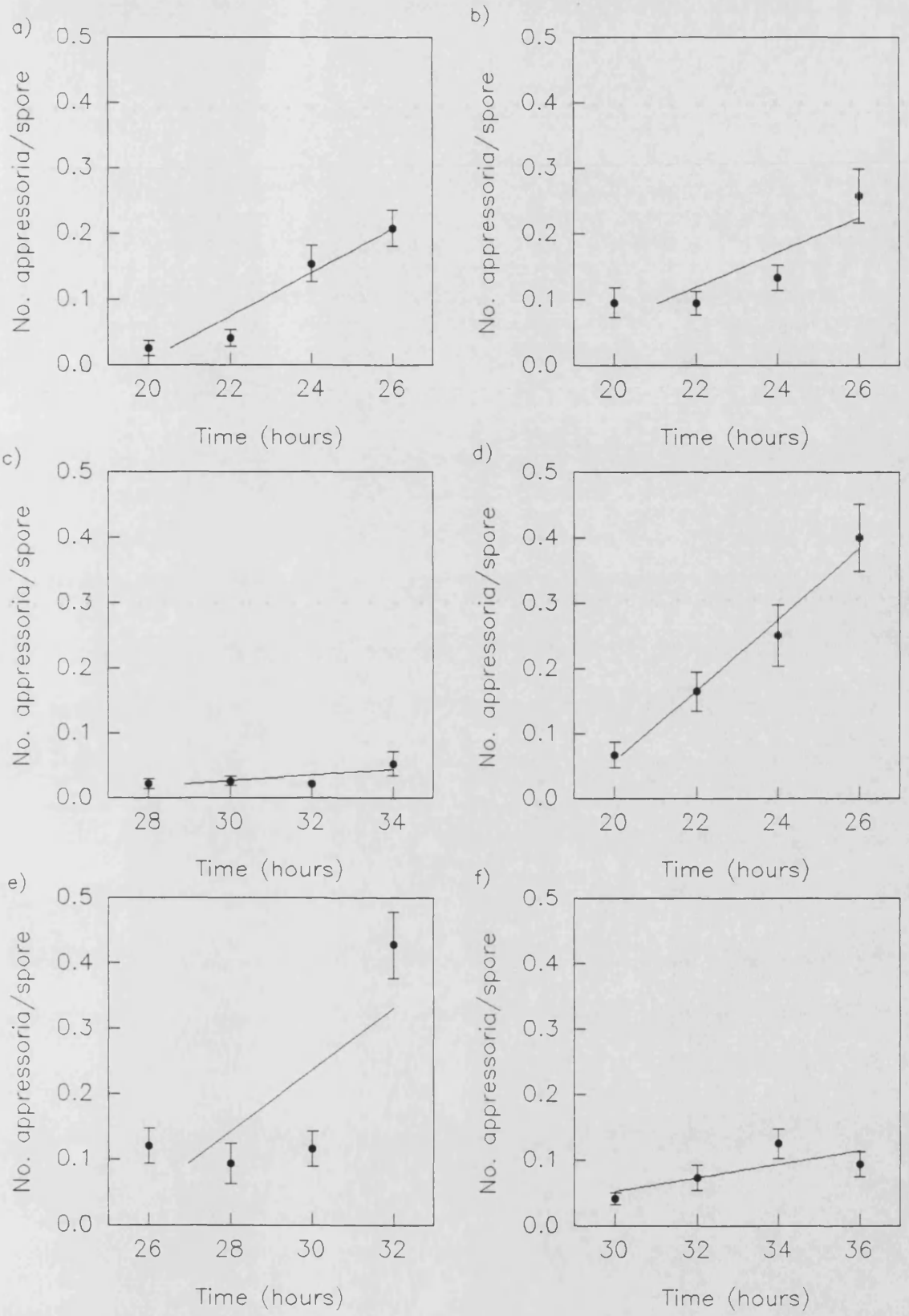
Mean, minimum and maximum percentage germination of spores of six Metarhizium anisopliae isolates, 6, 8, 10 and 12 hours post inoculation, incubated at 27°C on excised locust wings. Isolates a) Arsef 438, b) Arsef 439, c) Arsef 440, d) 191 633, e) 152222 and f) 298059. Regression lines have been fitted to the data.



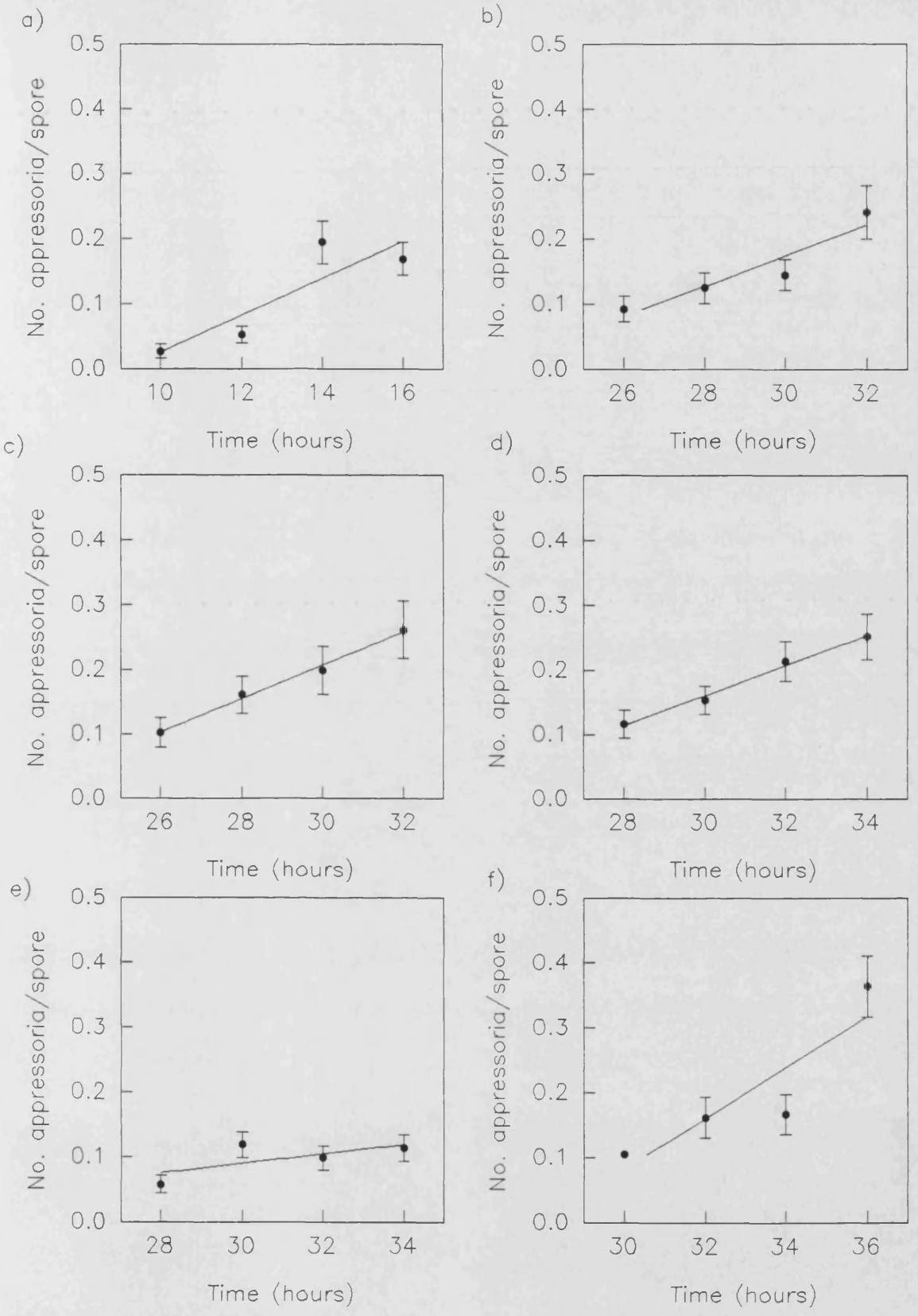
Mean, minimum and maximum percentage germination of spores of the Metarhizium anisopliae isolate I90 574, 6, 8, 10 and 12 hours post inoculation, incubated at 27°C on excised locust wings. A regression line has been fitted to the data.



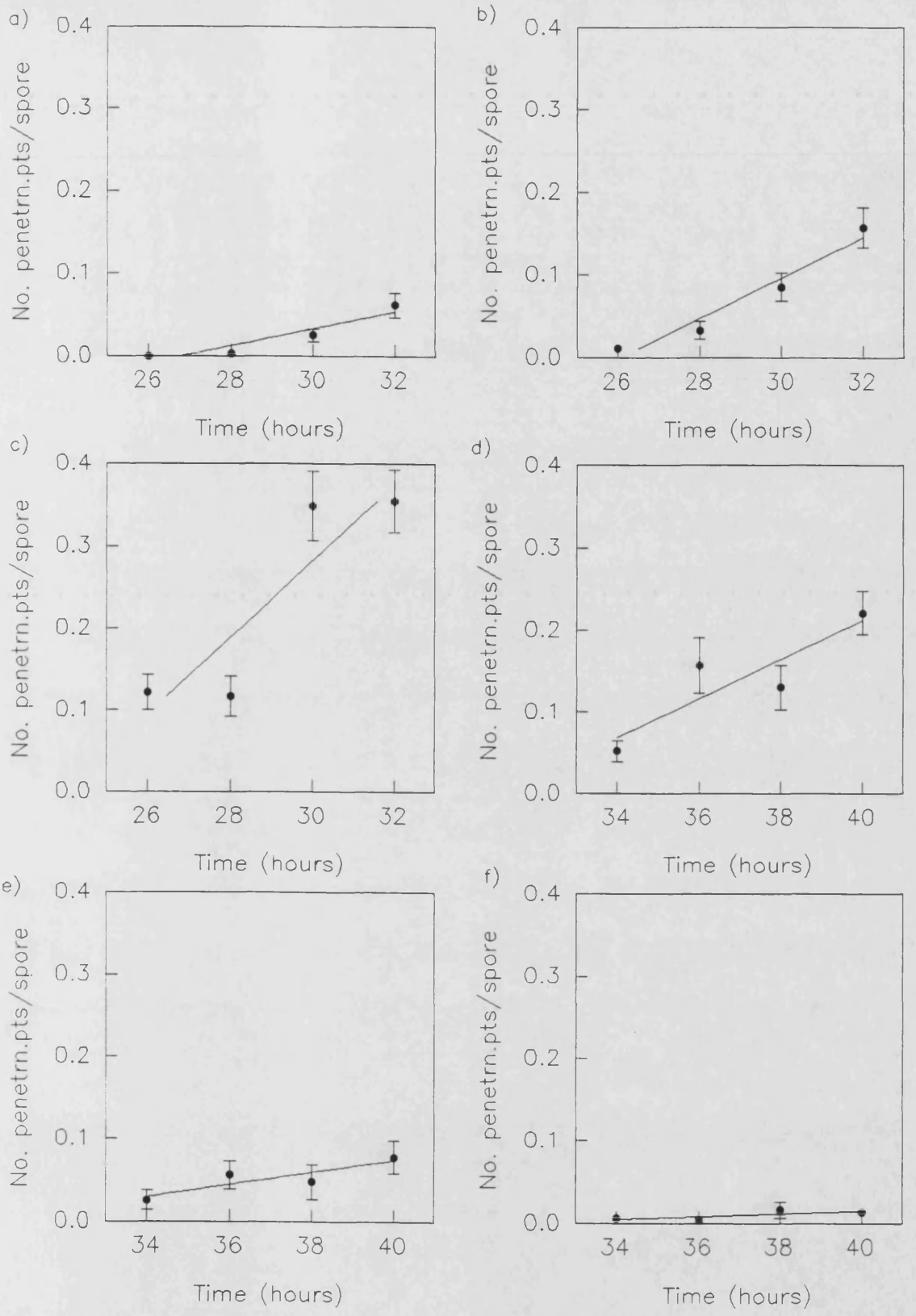
Number of appressoria/spore ± SE for six Metarhizium anisopliae isolates on excised locust wings, incubated at 27°C. Regression lines have been fitted to the data. a) Me1, b) 299981, c) 298061, d) I91 676, e) Arsef 727 and f) I90 574.



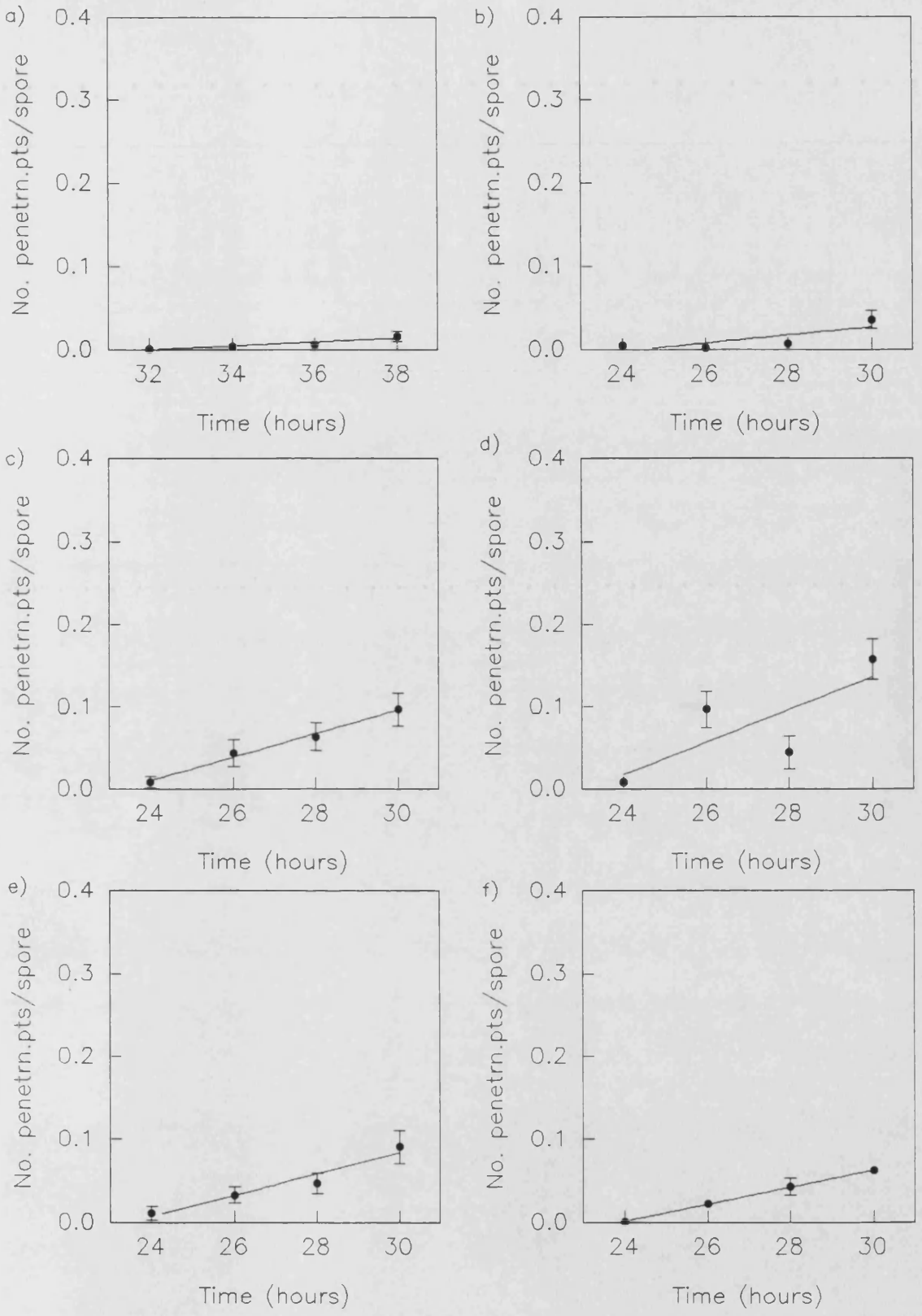
Number of appressoria/spore ± SE for six Metarhizium isolates on excised locust wings, incubated at 27°C. Regression lines have been fitted to the data. M. anisopliae isolates a) Arsef 324, c) 299984, d) 298059 and f) 191 633, and M. flavoviride isolates b) 324673, e) 330189.



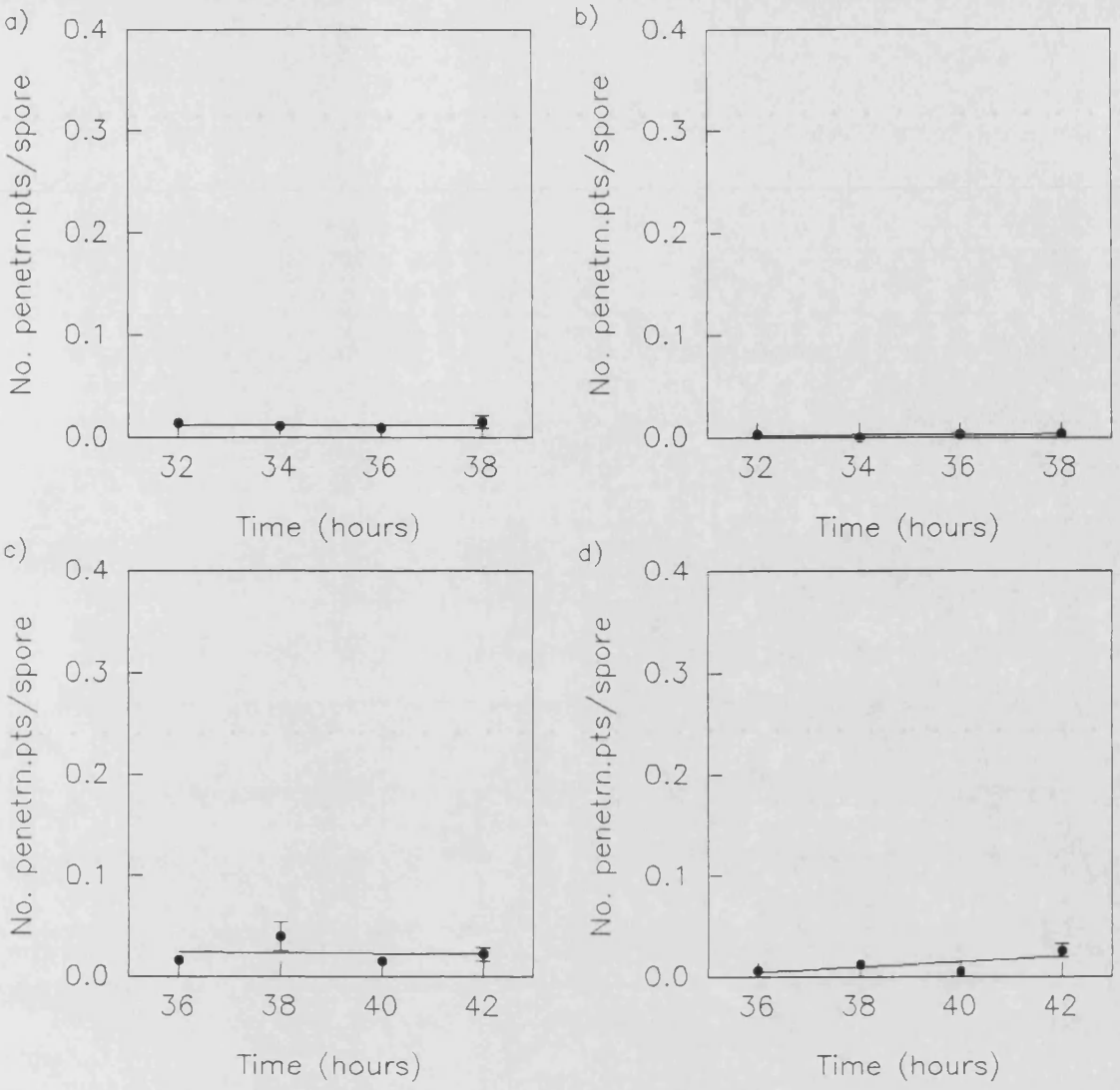
Number of appressoria/spore ± SE for six Metarhizium anisopliae isolates on excised locust wings, incubated at 27°C. Regression lines have been fitted to the data. a) 168777ii, b) 152222, c) Nr48, d) Arsef 440, e) Arsef 439 and f) Arsef 438.



Number of penetration points/spore ± SE for six Metarhizium anisopliae isolates on excised locust wings, incubated at 27°C. Regression lines have been fitted to the data. a) Arsef 324, b) Arsef 727, c) 298061, d) 298059, e) Arsef 438 and f) Nr48.



Number of penetration points/spore ± SE for six Metarhizium anisopliae isolates on excised locust wings, incubated at 27°C. Regression lines have been fitted to the data. a) 299984, b) 299981, c) 168777ii, d) 191 676, e) Me1 and f) 190 574.



Number of penetration points/spore ± SE for four Metarhizium anisopliae isolates on excised locust wings, incubated at 27°C. Regression lines have been fitted to the data. a) 152222, b) Arsef 440, c) Arsef 439 and d) 191 633.

APPENDIX 111

Bioassays:

Bioassays were carried out by the International Institute of Biological Control (IIBC), Silwood Park, Ascot, Berks.

Adult *Schistocerca gregaria* of either sex (in equal numbers where possible), 9 - 12 days after ecdysis, were used. Locusts were inoculated under the pronotum with 2 µl of a suspension containing 3.75×10^7 conidia/ml in cotton seed oil, giving 75,000 spores/locust. Controls were inoculated with oil alone. 25 locusts were used for each isolate. Locusts were then incubated individually in plastic food boxes (not airtight), with a piece of dry tissue paper in the bottom, at 30°C, 35% RH, in a 12 h light : 12 h dark regime. The locusts were denied access to food or water and mortality was recorded every day over the next 12 days. The percentage mortality for each isolate is recorded in the table over-leaf.

The median lethal time (MLT) was calculated by a computer program designed by Dr. R. Bateman, IIBC, Silwood Park, Ascot, Berks. MLT values could only be calculated for isolates that reached 50% mortality (12 out of 25 locusts) by day 12. For isolates that did not reach this level of mortality (I91 633, Arsef 438, Arsef 439 and Arsef 440) the MLT value would be greater than 12 but could not be calculated and therefore these four isolates were assigned the MLT value of 13.

Table of percentage mortality of adult *S. gregaria* inoculated with each of nineteen *Metarhizium anisopliae* and *flavoviride* isolates. The median lethal times (MLT) are also given.

ISOLATE	% DEAD ON DAY										MLT
	3	4	5	6	7	8	9	10	11	12	
330189	0	10	77	100	100	100	100	100	100	100	4.41
Arsef 324	14	62	96	100	100	100	100	100	100	100	3.71
Nr48	0	0	60	96	100	100	100	100	100	100	4.83
Arsef 2023	4	16	36	72	96	100	100	100	100	100	5.39
I91 676	0	0	0	48	92	96	100	100	100	100	6.05
168777ii	2	19	53	87	90	90	94	94	100	100	4.96
324673	6	10	78	84	84	100	100	100	100	100	4.59
Me 1	0	4	60	72	84	84	84	84	100	100	4.82
152222	2	8	40	70	84	84	88	88	88	88	5.48
299981	2	8	20	38	64	64	64	72	72	74	9.47
299984	0	0	24	42	50	60	60	66	66	66	9.03
298059	0	4	6	18	44	46	48	92	92	92	8.08
298061	0	0	4	16	40	64	72	88	88	96	7.42
Arsef 727	4	8	12	20	28	36	36	48	52	66	10.50
I91 633	0	4	16	20	20	20	20	32	32	32	13
I90 574	0	0	0	8	12	12	12	40	44	64	11.12
Arsef 438	4	4	4	4	4	4	4	4	4	20	13
Arsef 440	0	0	0	0	0	0	0	0	20	20	13
Arsef 439	0	0	0	0	0	0	0	0	0	6	13
control	0	0	0	0	0	0	0	5	25	30	-

APPENDIX IV

Manduca saline

Composition

Na^+	5.5 mM	K^+	40 mM
Mg^{2+}	18 mM	Ca^{2+}	3 mM
Cl^-	65 mM	Sucrose	193 mM
Phosphate	1.5 mM		

Stock solutions

10 x salt solution (1 litre)

NaCl	2.34 g	$MgCl_2.6H_2O$	36.58 g
KCl	29.84 g	$CaCl_2$	3.33 g
		(or $CaCl_2.2H_2O$	4.41 g)

100 x buffer solution

150 mM Na_2HPO_4	11.70 g	(500 ml)
150 mM NaH_2PO_4	10.65 g	(500 ml)

solutions mixed together on pH meter to pH 6.9

Making up Manduca *saline*

100 ml 10 x salt solution

10 ml 100 x buffer solution

66 g sucrose

1 litre distilled water

adjust to pH 6.5 using KOH

Ephrussi and Beadle's Saline

Composition of final saline

Na^+	128 mM
K^+	5 mM
Ca^{2+}	2 mM
Cl^-	135 mM

10x stock solution

NaCl	75 g
KCl	3.5 g
$CaCl_2$	2.1 g
(or $CaCl_2.2H_2O$	2.8 g)

Make up to 1 l in distilled water.

Dilute as required.

APPENDIX V

Bioassay results for male and female locusts inoculated with 75,000 spores per locust of *Metarhizium anisopliae* isolate Me1, in a 2 µl drop of cotton seed oil placed under the pronotum. Controls were inoculated with oil alone, in the same manner. The locusts were kept in individual containers, with no access to food or water, at 28°C, 30% RH with a 16 h light : 8 h dark cycle, and mortality was assessed daily.

	Infected females (24)		Control females (24)		Infected males (13)		Control males (13)	
Day	No. dead	Total dead	No. dead	Total dead	No. dead	Total dead	No. dead	Total dead
4	2	2	-	-	2	2	-	-
5	14	16	-	-	9	11	-	-
6	7	23	2	2	2	13	2	2
7	1	24	-	2	-	13	-	2

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Inhibition of Desert Locust (*Schistocerca gregaria*) Malpighian Tubule Fluid Secretion by Destruxins, Cyclic Peptide Toxins from the Insect Pathogenic Fungus *Metarhizium anisopliae*

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Received 14 December 1992; revised 4 February 1993

Destruxins are cyclic peptide lactone toxins isolated from the insect pathogenic fungus *Metarhizium anisopliae*. Destruxins A, A_2, B and E all inhibit fluid secretion *in vitro* by Malpighian tubules of the desert locust *Schistocerca gregaria*. Inhibition is dose-dependent; the IC_{50} for destruxin A is 23 μM. Destruxins A_2, B and E are similar to destruxin A in their effectiveness on fluid secretion at a concentration of 16 μM. Following a brief exposure to destruxin A *in vitro*, the rate of fluid secretion recovers significantly but incompletely. Fluid secretion was increased to 2.2 times the basal rate when Malpighian tubules were exposed to synthetic *Locusta migratoria* diuretic peptide. This stimulation of fluid secretion was completely inhibited by destruxin A. Fluid secretion by Malpighian tubules was stimulated by the intracellular second messenger, adenosine 3′,5′-cyclic monophosphate (cAMP), in the presence of the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX). This stimulation was also abolished by destruxin A. *Schistocerca* Malpighian tubules continued to secrete fluid in calcium-free conditions (zero calcium saline with added EGTA). Destruxin A inhibited fluid secretion equally well in the absence or presence of external calcium. The calcium channel blocker cadmium chloride did not prevent inhibition of fluid secretion by destruxin A nor did the anion channel blocker, 4-acetamido-4′-isothio-cyanatostilbene-2,2′-disulphonic acid (SITS). It is suggested that the inhibition by destruxin A of desert locust Malpighian tubule fluid secretion involves a cellular mechanism beyond the level of control by calcium or cAMP.

Desert locust *Schistocerca gregaria* Malpighian tubule Fluid secretion Cyclic peptide Toxin Destruxin *Metarhizium anisopliae* Diuretic peptide

INTRODUCTION

The deuteromycete fungus *Metarhizium anisopliae* is pathogenic to a wide range of insects including the desert locust, *Schistocerca gregaria* (Roberts and Humber, 1981). *M. anisopliae* produces a family of cyclic peptide lactone toxins, destruxins (Kodaira, 1962; Pais *et al.*, 1981; Gupta *et al.*, 1989), which may play a role in pathogenesis (Suzuki *et al.*, 1971; Samuels *et al.*, 1988a). The acute toxicity of destruxins to insects varies according to the species of insect tested, Lepidoptera and Diptera being particularly susceptible (Roberts, 1980; Samuels *et al.*, 1988a).

The toxins were originally isolated on the basis of their ability to cause immediate paralysis of silkworms (Kodaira, 1961). This effect in lepidopteran muscle is due to calcium-dependent depolarization of muscle cell membranes (Samuels *et al.*, 1988b). However, not all insects are susceptible to destruxin-induced paralysis, and other toxic effects of destruxins may also be important during *Metarhizium* mycosis. Vey and Quiot (1989) observed that the Malpighian tubules of wax-moth larvae, *Galleria mellonella*, were particularly sensitive to the cytotoxic effects of destruxins, showing adverse effects on ultrastructure after injection of destruxins.

Now that *M. anisopliae* is being considered as a possible biological control agent for desert locusts (Prior, 1992), it is of particular interest to investigate the effects of destruxins on *S. gregaria*. While destruxins are not toxic to *S. gregaria* when tested acutely (Samuels *et al.*, 1988a), Huxham *et al.* (1989) have shown that injections of destruxins depress the cellular immune

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responses of desert locusts. In this paper we describe the effects *in vitro* of a number of pure destruxins on the function of the Malpighian tubules of *S. gregaria*.

MATERIALS AND METHODS

Insects

Desert locusts (*S. gregaria* Forsk.) were from our own breeding culture at the University of Bath, U.K. They were reared as described previously (St Leger *et al.*, 1986), with the modification that the bran was sprayed with a solution of sodium sulphamethazine (4.26% w/v), sodium sulphathiazole (3.65% w/v) and sodium sulphamerazine (3.13% w/v) for control of *Malamoeba locustae* (Henry and Oma, 1968). Only freshly fed adult female locusts between 4 and 14 days after adult ecdysis were used for experiments.

Destruxins

These were obtained from culture filtrates of *Metarhizium anisopliae* (isolate Me 1). Briefly, the fungus was grown for 14 days in shake cultures on Czapek–Dox liquid modified medium with 0.5% Bacteriological Peptone. The medium was extracted in carbon tetrachloride, lyophilized, redissolved in propan-2-ol, lyophilized again, and then redissolved in acetonitrile before separation of destruxins by isocratic reverse phase HPLC on a Spherisorb C18 column using 60% acetonitrile as mobile phase. Destruxins were lyophilized and stored dry in the freezer until needed. Individual pure destruxins were identified by comparison of retention time with pure standards, and by fast-atom bombardment mass spectrometry (FAB-MS). Destruxins were dissolved directly in locust saline prior to testing.

Other chemicals

Synthetic *Locusta migratoria* diuretic peptide [sequence given by Kay *et al.* (1991)] was a generous gift from Dr G. M. Coast, Birkbeck College, University of London, U.K. The peptide was diluted in locust saline from a stock solution (3.8 μM) in methanol. The final concentration of methanol was 1%. Control saline included methanol but no peptide. Other chemicals used were from Sigma.

Fluid secretion

The rate of production of primary urine by *Schistocerca* Malpighian tubules *in vitro* was measured as originally described by Maddrell and Klunsuwan (1973), and modified by Anstee and Bell (1975). Briefly, the gut and associated Malpighian tubules were removed intact from the locust and maintained in a temperature-controlled bath (28°C), bathed in locust saline solution [composition given by Maddrell and Klunsuwan (1973)], the whole preparation being under the surface of a liquid paraffin (mineral oil) bath. In each preparation 10 Malpighian tubules were gently pulled out of the saline solution into the oil and looped around a peg. An incision made in each of these tubules allowed the escape of primary urine into a spherical droplet in the oil. Not all of the tubule loops secreted fluid; those that did not were ignored in subsequent analysis. Where a droplet was formed, its diameter was measured at intervals using an eyepiece graticule in a dissecting microscope in order to calculate its volume. Measurements were made on a number of droplets at 5 min intervals over a 40 min observation period, following which the saline solution in the bath was replaced. Rates of fluid secretion were determined from linear regressions of the volumes of droplets against time. For each treatment, mean rates of fluid secretion were calculated from a minimum of 15 tubules taken from two to four locusts.

It was not possible to ensure complete exchange of old medium for new when replacing the saline solution bathing the gut and Malpighian tubules. The efficiency of exchange was determined in a separate experiment with the dye, Trypan blue, to be 80%. Concentrations of chemicals quoted in this paper have been corrected for this factor.

RESULTS

Inhibition of fluid secretion by destruxin A

Schistocerca Malpighian tubules secreted primary urine *in vitro* at a mean rate of 5.71 ± 0.65 nl min^{-1} during an initial 40 min experimental period, in which the rate of secretion of individual tubules remained constant [Fig. 1(A)]. Following a change of the bathing medium and a 20 min acclimation period the rate was slightly but not significantly less (4.53 ± 0.54 nl min^{-1}).

When tubules were incubated according to the same protocol, first in locust saline solution and subsequently in locust saline containing destruxin A, the rate of secretion by the toxin-treated tubules was decreased. An example of a single tubule exposed to 80 μM destruxin A is shown in Fig. 1(B). The rate of fluid secretion by tubules exposed to the toxin was reduced, but constant during the 40 min experimental period. Inhibition of fluid secretion was dose-dependent (Fig. 2). The concentration of destruxin A required to give 50% inhibition (IC_{50}) was estimated to be 23 μM after allowing for the approx. 10% decrease in secretion that occurred during the experimental period even when destruxin was absent.

Activity of other destruxins

Four pure destruxins were tested. All inhibited fluid secretion to a similar extent (Fig. 3). There were no significant differences between the secretory rates of tubules exposed to the four toxins at a concentration of 16 μM (ANOVA, $F = 0.64$, $P = 0.7$, d.f. = 69).

Partial reversibility of destruxin inhibition

To assess the reversibility of the inhibition of fluid secretion by destruxin A, Malpighian tubules were

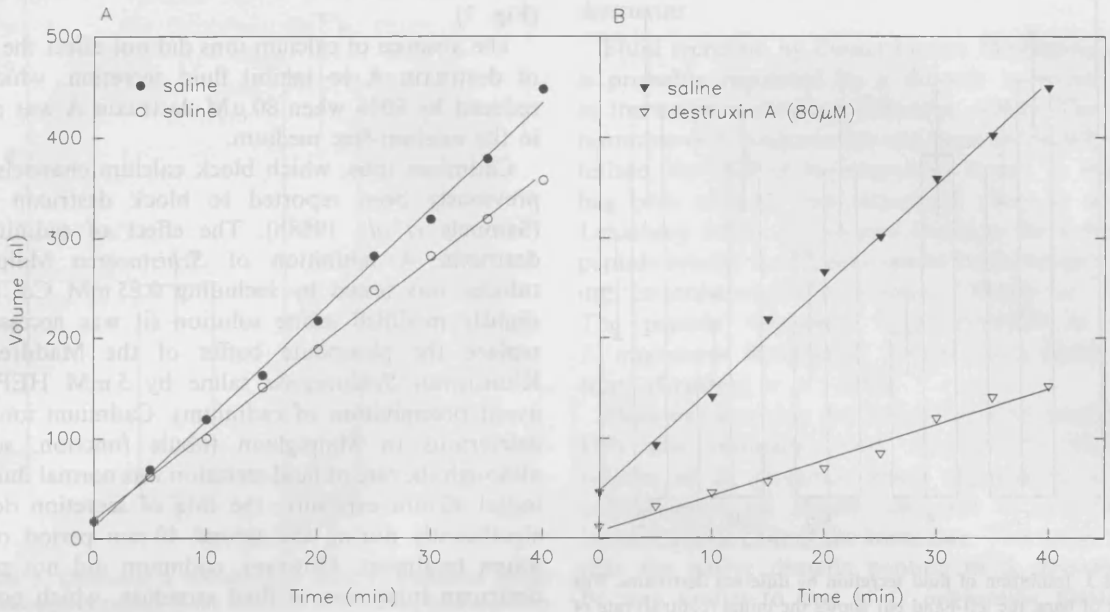

FIGURE 1. Fluid secretion by *Schistocerca* Malpighian tubules is inhibited by destruxin A. Each panel shows the cumulative secretion of fluid by a single Malpighian tubule with time. Solid symbols (●, ▼) show initial control experiments in which the tubule was bathed in locust saline. Open symbols show (A) a control experiment in which the tubule was again bathed in saline (○); (B) inhibition by 80 μM destruxin A (▽).

incubated first in locust saline solution (30 min), then exposed to 80 μM destruxin A (30 min), subsequently washed twice in destruxin A-free saline, then incubated for 30 min in saline solution (Fig. 4). A significant recovery in the rate of fluid secretion was observed when destruxin was removed, although the rate of secretion was significantly less than it had been prior to the toxin treatment. Additional washes did not lead to improved recovery (data not shown).

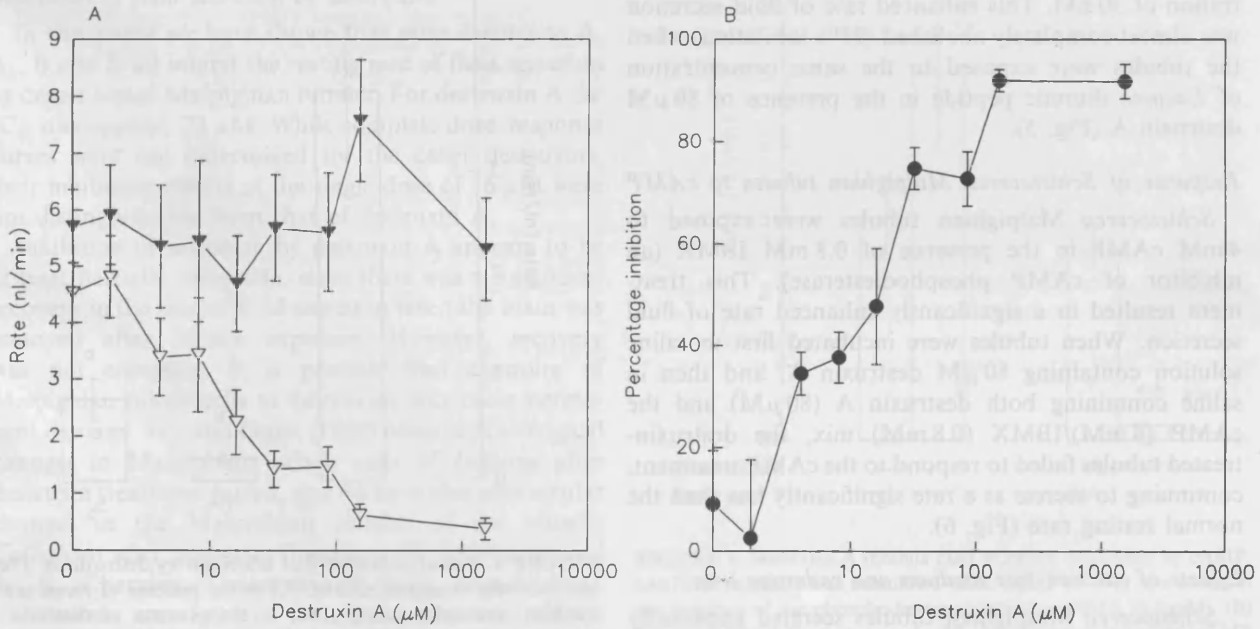

FIGURE 2. Dose–response relationship for inhibition of fluid secretion by destruxin A. (A) Rates of fluid secretion before (▼) and after (▽) application of destruxin A. (B) Percentage inhibition calculated from the data in (A). Means ± SE 16–22 tubules were used for each point. Each tubule was used to determine a control (saline) rate of secretion, and was then exposed to only a single concentration of destruxin A.

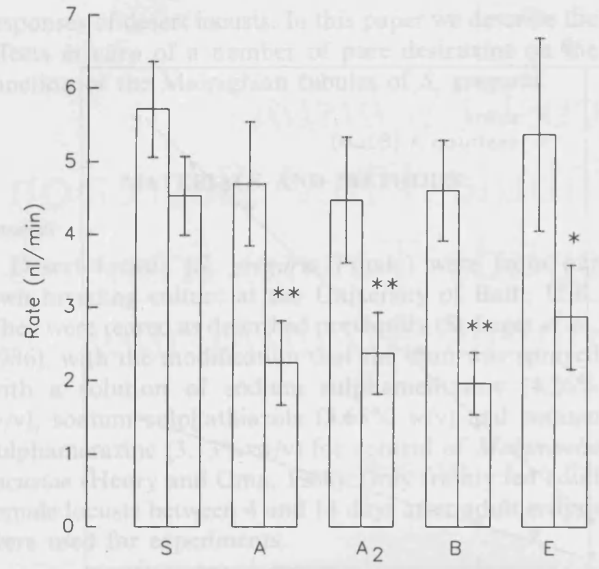

FIGURE 3. Inhibition of fluid secretion by different destruxins. For each pair of bars, the left-hand bar shows the initial (control) rate of fluid secretion in the presence of locust saline. The right-hand bar of each pair shows the (test) rate of secretion in the presence of (S) replacement locust saline; (A) destruxin A (16 μM); (A_2) destruxin A_2 (16 μM); (B) destruxin B (16 μM); (E) destruxin E (16 μM). Means $\pm$ SE 16–19 tubules in each case. For each treatment the initial (control) rate was compared with the subsequent test using a paired t-test. $*P < 0.05$; $**P < 0.01$.

Response of Schistocerca Malpighian tubules to Locusta diuretic peptide

Locusta diuretic peptide significantly increased the rate of fluid secretion *in vitro* by *Schistocerca* Malpighian tubules when added to the bathing medium at a concentration of 30 nM. This enhanced rate of fluid secretion was almost completely abolished (93% inhibition) when the tubules were exposed to the same concentration of *Locusta* diuretic peptide in the presence of 80 μM destruxin A (Fig. 5).

Response of Schistocerca Malpighian tubules to cAMP

Schistocerca Malpighian tubules were exposed to 4 mM cAMP in the presence of 0.8 mM IBMX (an inhibitor of cAMP phosphodiesterase). This treatment resulted in a significantly enhanced rate of fluid secretion. When tubules were incubated first in saline solution containing 80 μM destruxin A, and then in saline containing both destruxin A (80 μM) and the cAMP (4 mM)/IBMX (0.8 mM) mix, the destruxin-treated tubules failed to respond to the cAMP treatment, continuing to secrete at a rate significantly less than the normal resting rate (Fig. 6).

Effects of calcium-free medium and cadmium ions

Schistocerca Malpighian tubules secreted apparently normally in the absence of external calcium. When the tubules were bathed in a modified locust saline without calcium salts and containing 1 mM EGTA, the rate of fluid secretion was similar to the normal basal (unstimulated) rate over two successive 40 min periods (Fig. 7).

The absence of calcium ions did not affect the ability of destruxin A to inhibit fluid secretion, which was reduced by 80% when 80 μM destruxin A was present in the calcium-free medium.

Cadmium ions, which block calcium channels, have previously been reported to block destruxin action (Samuels *et al.*, 1988b). The effect of cadmium on destruxin A inhibition of *Schistocerca* Malpighian tubules was tested by including 0.25 mM $CdCl_2$ in a slightly modified saline solution (it was necessary to replace the phosphate buffer of the Maddrell and Klunsuwan *Schistocerca* saline by 5 mM HEPES to avoid precipitation of cadmium). Cadmium ions were deleterious to Malpighian tubule function, so that although the rate of fluid secretion was normal during an initial 40 min exposure, the rate of secretion declined significantly during the second 40 min period of cadmium treatment. However, cadmium did not prevent destruxin inhibition of fluid secretion, which occurred normally (Fig. 7).

Effects of SITS

Cerenius *et al.* (1990) reputed that the stimulatory effects of destruxin A on the degranulation of crayfish haemocytes were abolished by the anion channel blocker, SITS. At 8 mM, a similar concentration to that used by Cerenius *et al.* (1990), this chemical proved to be an inhibitor of resting fluid secretion in *Schistocerca* Malpighian tubules [see Fig. 8(A)]. However, even at

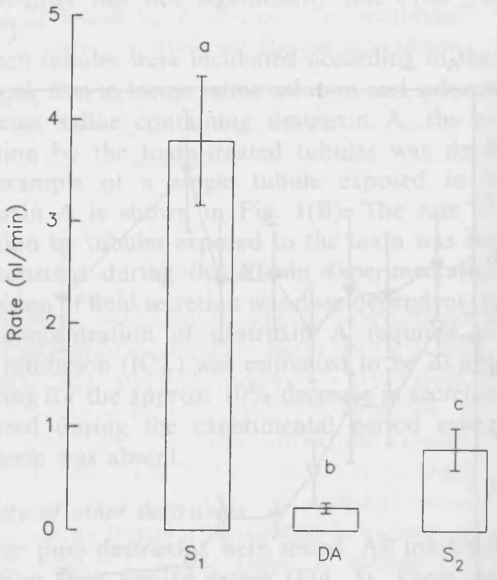

FIGURE 4. Partial reversibility of inhibition by destruxin A. Fluid secretion was measured initially (S_1) in the presence of locust saline (30 min), and subsequently (DA) in the presence of destruxin A (80 μM) (30 min), at the end of which time the tubules were washed twice with locust saline. Fluid secretion was now measured in the presence of toxin-free locust saline (S_2) (30 min). Mean $\pm$ SE 21 tubules were used in each case. Rates were compared using a paired t-test. Significant differences ($P < 0.01$) are indicated by different letters.

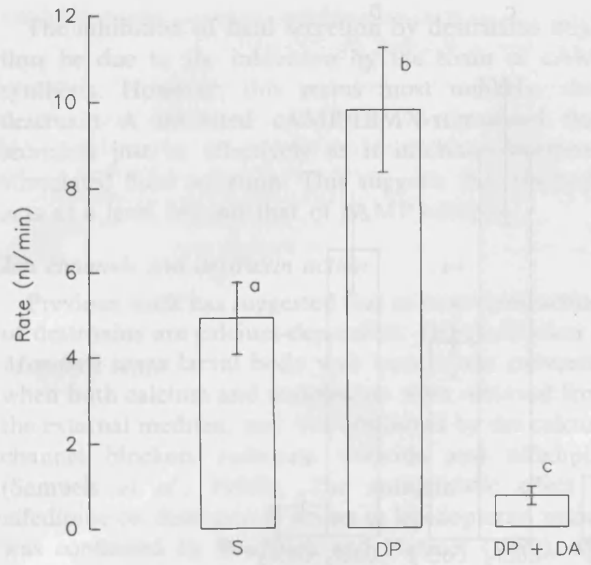

FIGURE 5. Destruxin A inhibits fluid secetion stimulated by *L. migratoria* diuretic peptide (Lom DP). For each tubule, fluid secretion was measured during successive 30 min periods in the presence of (S) locust saline, (DP) Lom DP (30 nM), and (DP + DA) Lom DP (30 nM) and destruxin A (80 μM). Mean $\pm$ SE 18 tubules used in each case. Rates were compared using a paired t-test. Significant differences ($P < 0.01$) are indicated by different letters.

this concentration, SITS failed to prevent the further inhibition of fluid secretion by 80 μM destruxin A [Fig. 8(B)].

DISCUSSION

Inhibition of fluid secretion by destruxins

In this paper we have shown that pure destruxins A, A_2, B and E all inhibit the resting rate of fluid secretion by desert locust Malpighian tubules. For destruxin A the IC_{50} was approx. 23 μM. While complete dose–response curves were not determined for the other destruxins, their inhibitory effects at the single dose of 16 μM were not distinguishable from that of destruxin A.

Inhibition of secretion by destruxin A appears to be at least partially reversible, since there was a significant recovery in the rate of fluid secretion when the toxin was removed after 30 min exposure. However, recovery was not complete. It is possible that exposure of Malpighian tubule cells to destruxins may cause permanent damage. Vey and Quiot (1989) observed cytological changes in Malpighian tubule cells of *Galleria* after destruxin treatment *in vivo*, and we have also seen similar changes in the Malpighian tubules of the blowfly *Calliphora* after destruxin injections (S. R. Watson and A. K. Charnley, University of Bath, unpublished). Destruxins are known to have cytotoxic effects on cultured insect cells (Vey and Quiot, 1989; M. J. Kershaw, unpublished). It is not clear whether these cytotoxic effects of destruxins are directly related to the inhibition of fluid secretion described here.

Stimulation of fluid secretion and its inhibition by destruxins

Fluid secretion by Desert Locust Malpighian tubules is probably regulated by a diuretic hormone present in the corpora cardiaca (Mordue, 1969). The diuretic hormone of *S. gregaria* has not been chemically characterized, but that of the migratory locust, *L. migratoria* has been isolated and sequenced (Kay *et al.*, 1991; Lehmberg *et al.*, 1991), and found to be a 46-residue peptide related in sequence to the corticotrophin releasing factor/sauvagine/urotensin I family of peptides. The peptide stimulates fluid secretion in isolated *L. migratoria* Malpighian tubules by a factor of 2–3 times (Lemberg *et al.*, 1991).

Here we show that the *Locusta* diuretic peptide (Lom DP) also stimulates fluid secretion by Malpighian tubules of *S. gregaria*. When exposed to a peptide concentration of 30 nM, the rate of secretion was increased to 2.2 times the basal rate. This result suggests that the native diuretic peptide in *S. gregaria* must be very similar to that of *L. migratoria*. Destruxin A inhibited Lom DP-stimulated fluid secretion. Eighty μM toxin caused a 93% reduction in secretory rate as compared to the stimulated control.

Mechanism of action of destruxins

Lom DP probably stimulates fluid secretion by increasing the production of the intracellular second messenger cAMP in Malpighian tubule cells (Morgan

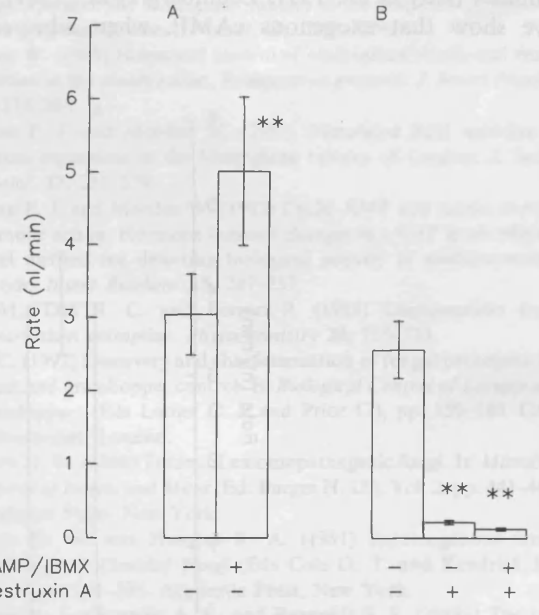

FIGURE 6. Destruxin A inhibits fluid secretion stimulated by cAMP. (A) Fluid secretion was stimulated when cAMP (4 mM) was added in the presence of the phosphodiesterase inhibitor IBMX (0.8 mM). (B) Preincubation with destruxin A (80 μM) (30 min) prevented cAMP stimulation of fluid secretion. Means $\pm$ SE 22–23 tubules in each case. Rates were compared using a paired t-test. Significant differences from the control (i.e. no cAMP/IBMX, no destruxin A) are indicated ($^{**}P < 0.01$).

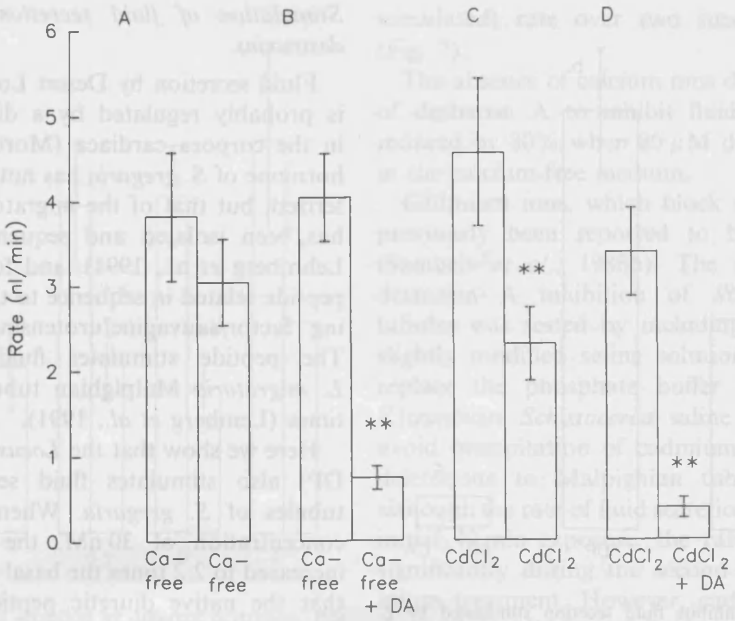

FIGURE 7. Calcium, cadmium and the action of destruxin A. (A) Calcium-free locust saline (containing 1 mM EGTA) does not significantly inhibit fluid secretion; (B) 80 μM destruxin A (DA) inhibits fluid secretion in the absence of external calcium; (C) cadmium chloride (0.25 mM) in calcium-free locust saline (without EGTA) significantly inhibits the resting rate of fluid secretion; (D) in the presence of 0.25 mM cadmium chloride, destruxin A (80 μM) causes further significant inhibition of fluid secretion. [N.B. In experiments (C) and (D) the locust saline was buffered with HEPES instead of sodium phosphate.] Means $\pm$ SE 16–24 tubules in each case. Experimental treatments were compared with the corresponding control using a paired t-test. $^{**}P < 0.001$.

and Mordue, 1985). Synthetic Lom DP is known to increase cAMP levels in *L. migratoria* Malpighian tubules (Kay *et al.*, 1991; Lehmberg *et al.*, 1991). Here we show that exogenous cAMP, when administered together with the cAMP phosphodiesterase inhibitor IBMX, causes an increased rate of fluid secretion in *S. gregaria* tubules just as has been shown in *L. migratoria* (Morgan and Mordue, 1981, 1985).

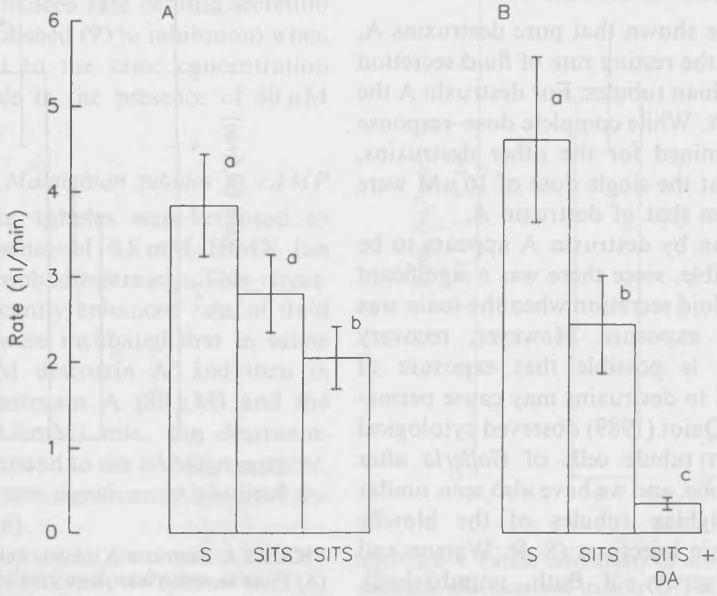

FIGURE 8. Effect of SITS on fluid secretion and on destruxin A action. (A) Malpighian tubules were incubated for successive 30 min periods with (S) locust saline, and subsequently with two changes of SITS (8 mM); (B) tubules were incubated successively with saline (S), SITS (8 mM) and SITS (8 mM) plus destruxin A (80 μM) (DA). Means $\pm$ SE (A) 23 tubules; (B) 15 tubules. Means were compared using a paired t-test. Significant differences ($P < 0.01$) within each experiment are indicated by different letters.

The inhibition of fluid secretion by destruxins might thus be due to the inhibition by the toxin of cAMP synthesis. However, this seems most unlikely, since destruxin A inhibited cAMP/IBMX-stimulated fluid secretion just as effectively as it inhibited hormone-stimulated fluid secretion. This suggests that the toxin acts at a level beyond that of cAMP control.

Ion channels and destruxin action

Previous work has suggested that at least some actions of destruxins are calcium-dependent. Depolarization of *Manduca sexta* larval body wall muscle was prevented when both calcium and magnesium were removed from the external medium, and was abolished by the calcium channel blockers cadmium chloride and nifedipine (Samuels *et al.*, 1988b). The antagonistic effect of nifedipine on destruxin B action in lepidopteran muscle was confirmed by Bradfisch and Harmer (1990), who also found that omega-conotoxin GVIA prevented destruxin-induced depolarization. These findings suggest that voltage-gated calcium channels may be involved in the response to destruxins. Destruxin-induced degranulation of crayfish (*Pacifastacus leniuseulus*) haemocytes is also calcium-dependent, being abolished in calcium-free conditions and by cadmium ions (Cerenius *et al.*, 1990).

In the present case, however, we did not find the inhibition of fluid secretion by destruxins to be calcium-dependent. Calcium-free conditions did not prevent fluid secretion continuing at normal basal rates, nor did they prevent inhibition by destruxin A. Cadmium chloride (used at a concentration adequate to prevent destruxin-induced depolarization in *M. sexta* muscle) itself caused some inhibition of fluid secretion. However, the use of appropriate controls clearly showed that cadmium ions did not prevent the destruxin inhibition of fluid secretion by *S. gregaria* Malpighian tubules, which occurred normally.

The degranulating effects of destruxins on crayfish haemocytes are also inhibited by the anion channel blocker, SITS (Cerenius *et al.*, 1990). We found that SITS alone caused a progressive reduction in the rate of fluid secretion by *S. gregaria* Malpighian tubules. Again, however, this did not prevent the normal inhibition of fluid secretion by destruxin A.

These two results suggest that neither calcium channels nor anion channels participate directly in the toxic effects of destruxins on Malpighian tubules, at least as far as fluid secretion is concerned. It also suggests that either the mechanism of action of destruxins in *S. gregaria* Malpighian tubules is different to that in caterpillar muscles and crayfish haemocytes, or that in the latter cases these ion channels are only indirectly involved in the expression of the symptoms of destruxin-induced toxicity. A similar conclusion was reached for the inhibition by destruxins of ecdysteroid secretion in *M. sexta* prothoracic glands (Sloman and Reynolds, 1993), where it was suggested that the toxins act at a level beyond the control of either calcium or cAMP.

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